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Infection of stem cell-derived hepatocytes with hepatotropic viruses

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Summary

Currently, *in vitro* cell culture models for the study of hepatotropic viruses rely on the use of primary human hepatocytes and hepatoma cell lines, which both have conceivable limitations. Alternative sources of human hepatocytes are human embryonic (hESCs) and induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells. These pluripotent stem cells (PSCs) have numerous advantages compared to primary cells, including the fact that they can theoretically self-renew indefinitely, can differentiate into any cell type of the human body and can relatively easily be genetically modified.

Previous studies from the Verfaillie lab demonstrated that stem cell-derived hepatocytes can be infected with the hepatitis C virus (HCV). Another more recently described virus that causes hepatitis is the hepatitis E virus (HEV). Every year 20 million people become infected with HEV causing over 3 million cases of acute hepatitis. Although hepatitis E is usually an acute self-limiting form of hepatitis, severe cases of fulminant hepatitis and chronic infections have been reported, causing an estimated 56.000 deaths per year. HEV research is severely hampered by the lack of efficient cell culture models that support HEV replication. In this thesis I differentiated hESCs and hiPSCs toward hepatocytes and subsequently infected the hepatic progeny cells with HEV. I demonstrated that stem cell-derived hepatocytes support the complete replication of the HEV. In addition, I found that pluripotent stem cells differentiated toward mesoderm or neural progenitor cells did not support HEV replication confirming the specific hepatocyte tropism of HEV. However, I demonstrated that both mesodermal cells and neuroprogenitors can support replication when the viral entry step was circumvented.

The hepatocyte differentiation protocol that was used to demonstrate the susceptibility of stem cell-derived hepatocytes to HEV consisted of a mixed population of cells. Therefore, I performed studies to improve hepatocyte differentiation from PSCs by the addition of dimethyl sulfoxide (DMSO). I demonstrated that addition of DMSO during hepatocyte differentiation induced a more homogenous population of PSC progeny. The improved PSC progeny homogeneity also enhanced HEV infection. Moreover, the expression of Natrium-taurocholate cotransporting polypeptide (NTCP), a key receptor

for HBV, was tremendously increased by the addition of DMSO. Because of the abundant NTCP expression, the DMSO differentiated hepatocyte progeny cells were also supporting infection with HBV, even if these studies will need further confirmation.

Finally, I examined whether PSC-derived hepatocytes could be infected with the zika virus (ZIKV). Recently, ZIKV gained attention because of birth defects associated with ZIKV infection in South America. However, ZIKV mouse models suggest not only high viral RNA titers in the brain but also in the liver. As a case report published in 1954 described the occurrence of liver damage and jaundice in two patients with elevated ZIKV viral antibodies, and ZIKV belongs to the *Flaviviridae* family, also encompassing Dengue virus and Yellow Fever virus, we tested if ZIKV can infect human PSC-hepatocytes. I demonstrated that hPSC-HLCs support the complete replication cycle of ZIKV, including entry, replication and production of infectious virions. I also demonstrated that treatment with 7-deaza-2'-*C*-methyladenosine (7DMA), a known (+)ssRNA virus inhibitor, inhibits ZIKV infection in a dose-dependent manner. Although clinical data about ZIKV infection are limited, these studies suggest that the liver might be a potential target for ZIKV and that certain strains could cause hepatitis and liver damage.

In conclusion, the studies in this thesis demonstrate that pluripotent stem cell-derived hepatocytes are a promising model to study infection with various hepatotropic viruses and, can be considered a novel test system for antiviral drugs.

Samenvatting

Momenteel worden primaire humane hepatocyten en hepatoma cellijnen gebruikt voor de *in vitro* studie van hepatocyt specifieke virussen. Het gebruik van deze systemen is echter gelimiteerd. Alternatieve bronnen van humane hepatocyten zijn humane embryonale (hESCs) en geïnduceerde pluripotente (hiPSCs) stamcel afgeleide hepatocyten. hESCs en hiPSCs hebben vele voordelen vergeleken met primaire humane hepatocyten zoals de capaciteit om zichzelf oneindig te hernieuwen, de potentie om te differentiëren in elk mogelijk cel type en de mogelijkheid om genetisch gemanipuleerd te worden.

Voorgaande studies hebben aangetoond dat stamcel afgeleide hepatocyten geïnfecteerd kunnen worden met het hepatitis C (HCV) en hepatitis B virus (HBV). Een ander, meer recent beschreven, virus dat hepatitis veroorzaakt, is het hepatitis E virus (HEV). Elk jaar worden 20 miljoen mensen geïnfecteerd met het hepatitis E virus wat resulteert in meer dan 3 miljoen gevallen van acute hepatitis. Hoewel Hepatitis E voornamelijk acute hepatitis veroorzaakt, zijn er ook gevallen van fulminante en chronische hepatitis gerapporteerd resulterend in ongeveer 56.000 doden per jaar. HEV onderzoek wordt belemmerd door het gebrek aan efficiënte celcultuur systemen die HEV replicatie ondersteunen. In deze thesis, differentieerde ik hESCs en hiPSCs tot hepatocyten en infecteerde deze met HEV. Ik toonde aan dat stamcel afgeleide hepatocyten de volledige replicatie cyclus van HEV ondersteunen. Bovendien vonden we dat stamcel afgeleide mesodermale cellen en neuroprogenitors HEV replicatie niet ondersteunen en HEV replicatie dus specifiek lijkt te zijn voor hepatocyten. Nochtans konden deze twee celtypes wel de replicatie van HEV ondersteunen wanneer de binnenkomst van het virus omzeild was.

Het hepatocyt differentiatie protocol dat gebruikt werd om de infectie met HEV aan te tonen resulteerde in een gemengde cel populatie. Ik streefde daarom ernaar het hepatocyt differentiatie protocol te verbeteren door het toevoegen van dimethyl sulfoxide (DMSO). Het toevoegen van DMSO zorgde voor een meer homogene populatie van stamcel afgeleide hepatocyten en een betere infectiviteit met HEV. Bovendien was de expressie van natrium-taurocholaat cotransporting polypeptide (NTCP) enorm verhoogd door de toevoeging van DMSO. Aangezien NTCP een cruciale HBV receptor is, was de stamcel afgeleide hepatocyt populatie nu ook susceptibel voor HBV.

Tot slot onderzocht ik of stamcel afgeleide hepatocyten ook geïnfecteerd konden worden met het zika virus (ZIKV). ZIKV werd recent in de media gebracht vanwege de mogelijke link met geboortedefecten. Nochtans tonen muis modellen aan dat ZIKV niet alleen gevonden kan worden in de hersenen maar ook in de lever. Verder werd er in 1954 een case rapport gepubliceerd waarbij er een mogelijke link zou zijn tussen ZIKV en geelzucht. Ik toonde aan dat stamcel afgeleide hepatocyten ook de volledige replicatie cyclus van ZIKV ondersteunen. Bovendien toonde ik aan dat ZIKV infectie geïnhibeerd kon worden met 7-deaza-2'-C-methyladenosine (7DMA); een gekende RNA virus inhibitor. Wij suggereren dus dat ZIKV mogelijks infectieus is voor de lever al is bijkomend klinisch onderzoek nodig om dit te bevestigen.

Samengevat tonen onze studies aan dat pluripotente stamcel afgeleide hepatocyten een veelbelovend model zijn voor de studie van verschillende hepatocyt specifieke virussen en gebruikt kunnen worden voor het evalueren van antivirale geneesmiddelen.

Abbreviations

(+)ssRNA	positive strand RNA
(-)ssRNA	negative strand RNA
αSMA	alpha smooth muscle actin
AAT	alpha 1-antitrypsin
AFP	alpha fetoprotein
ALB	albumin
ALT	alanine aminotransferase
ATF5	activating transcription factor 5
BAL	bio-artificial liver
BIO	6-bromoindirubin-3′-oxime
BLBP	brain lipid-binding protein
BMP4	bone morphogenetic protein 4
cccDNA	covalently closed circular DNA
COL1A1	collagen type 1 alpha 1
CYP450	cytochrome P450
DLX2	distal-less homeobox 2
DE	definitive endoderm
DEX	dexamethason
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EIF2AK2	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2
FBS	fetal bovine serum
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FOXA1	Forkhead Box A1
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HBV	hepatitis B virus
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma

hESC	human embryonic stem cells
HEV	hepatitis E virus
HGF	hepatocyte growth factor
hiPSC	human induced pluripotent stem cells
HBx	hepatitis B X protein
HLC	hepatocyte-like cell
HNF4α	hepatocyte nuclear factor 4
IFN	interferon alpha
iHEP	induced hepatocytes
iMEF	inactivated mouse embryonic mouse fibroblasts
ISG	interferon stimulated genes
JAK	janus kinase
LSEC	liver sinusoidal endothelial cells
LOX1	leech homeobox 1
NIM	neural induction medium
NMM	neural maintenance medium
NTCP	natrium-taurocholate cotransporting polypeptide
MyrB	myrcludex B
OD	optical density
OSM	oncostatin M
ORF	open reading frame
PAX6	Paired box protein Pax-6
PDGFRβ	beta-type platelet-derived growth factor receptor
PHH	primary human hepatocytes
PPAR	peroxisome proliferator- activated receptor
PROX1	Prospero Homeobox 1
PSC	pluripotent stem cells
PXR	pregnane X receptor
RBV	ribavirin
rcDNA	relaxed circular DNA
RdRp	RNA-dependent RNA polymerase
RIG1	retinoic acid-inducible gene 1
ROS	reactive oxygen species

qRT-PCR	quantitative reverse-transcription polymerase chain reaction
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SSEAA	stage-specific embryonic antigen-4
STAT	signal transducer and activator of transcription
STM	septum transversum
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TLR	toll-like receptor
uPA	urokinase plasminogen activator
UTR	untranslated region
VLP	virus like particle
ZIKV	ZIKA virus

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Chapter I: Introduction

1.1 Human hepatocytes: general introduction

1.1.1 Organization of the liver

The liver is the largest internal organ and is responsible for the major metabolic functions of the human body, including detoxification, metabolism of drugs and dietary nutrients, protein synthesis, production of albumin and bile and glycogen storage. All of these functions are executed by hepatocytes, the main liver cell type accounting for approximately 70% of the total liver mass (1, 2). Blood and nutrients enter the sinusoidal space of the liver via the portal vein and the hepatic artery and re-enter in the circulation through the central vein. The liver is zonated and the function of hepatocytes differs according to their location along the porto-central axis, which is associated with a decrease in oxygen level (Figure 1). Periportal hepatocytes are responsible for urea synthesis, gluconeogenesis and oxidative phosphorylation while perivenous hepatocytes manage glutamine synthesis and glycolysis. Other functions of the liver such as albumin secretion are however not subjected to zonation (3). In addition, hepatocytes are polarized cells with a basolateral and apical membrane that are distinct by their expression of various (drug) transporters. The apical membrane faces the bile canaliculi while the basolateral membrane is facing the sinusoidal space (2).

The other liver cell types; stellate cells and liver sinusoidal endothelial cells (LSECs), are derived from the embryonic mesoderm while kupffer cells originate from the yolk sac (4). The hepatocytes are organized in single cell layers and lined by the LSECs. Hepatic stellate cells reside in the space of Disse, which is the space between the hepatocytes and the LSECs (Figure 1) (2). Hepatic stellate cells represent approximately 8% of the liver tissue and remain quiescent in healthy liver. Upon liver damage, stellate cells become activated, secrete collagen and form a fibrotic scar that can eventually lead to irreversible liver damage and cirrhosis (5). The liver sinusoidal endothelial cells are characterized by pores or fenestrae, which is specific for endothelial cells residing in the liver. The fenestrae function as a sieve to filter the blood that arrives in the sinusoid through the portal vein before it is passed through the hepatocytes (6). In addition, LSECs function as scavengers of macromolecules and contribute to the maintenance of the liver homeostasis by the release of soluble mediators such as nitric oxide and pro-

inflammatory mediators (7, 8). Kupffer cells are present on the endothelial lining and function as the macrophages of the liver, playing important roles in inflammation by producing pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) (9).

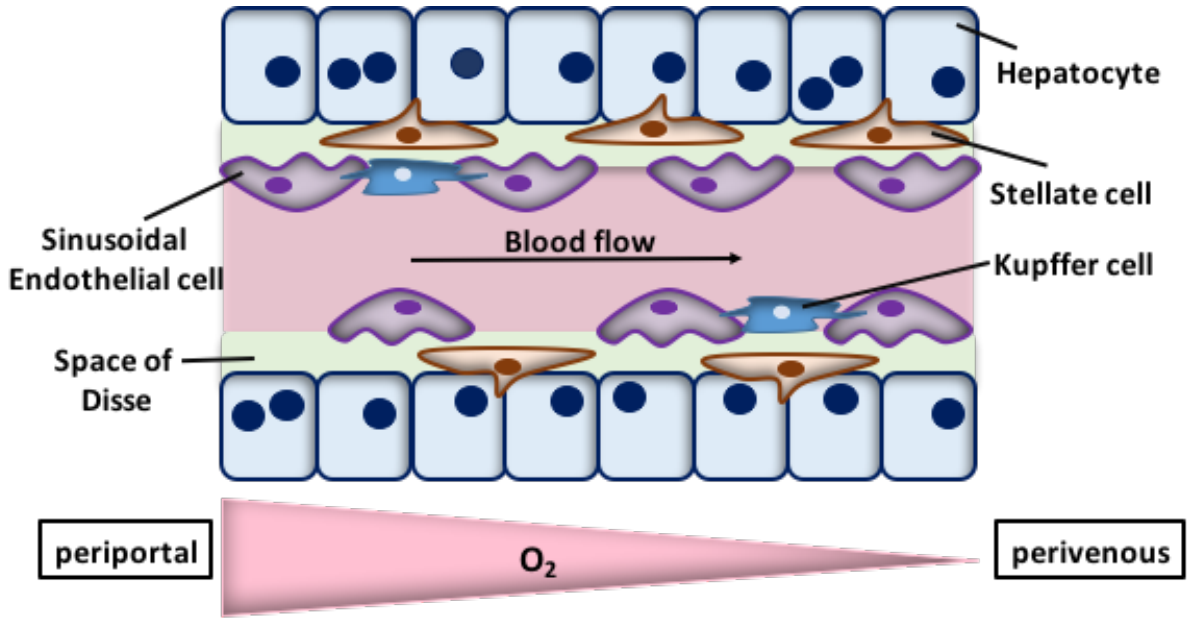

Figure 1. *Organization of the liver. Hepatocytes are lined by the liver sinusoidal endothelial cells with in between the hepatic stellate cells in the space of Disse. Kupffer cells are present in the sinusoids adjacent to the endothelial cells. Blood flows from the portal vein and hepatic arteriole to the central vein together with a decrease in oxygen concentration.*

1.1.2 Hepatocytes and the need for renewable sources

The liver is prone to many diseases caused by e.g. hepatitis viruses, drug intake, alcohol abuse or inheritable and auto-immune disorders such as alpha-1-antitrypsinedeficiency. In healthy liver, hepatocytes remain in a quiescent state but upon liver injury, hepatocytes are triggered to proliferate in order to restore and regenerate the damaged tissue (10). When liver damage proceeds stellate cells become activated and form a fibrotic scar, which evolves into cirrhosis. Both cirrhosis and uncontrolled hepatocyte proliferation can lead to hepatocellular carcinoma, the third leading cause of cancer deaths (11). The process of hepatocyte activation is still not completely understood. Expression of several liver-enriched transcription factors such as Hepatocyte Nuclear Factor 4 alpha (HNF4α) and the Wnt/β-catenin pathway are

thought to be involved in the control of hepatocyte proliferation and liver regeneration (10, 12). Because mature hepatocytes are characterized by a tremendous regeneration potential, the existence and role of liver stem cells or progenitor cells is still under debate. During recent years, studies were published both in favor or against the presence of resident stem cells in the liver (13-16). For instance, Lu *et al.* demonstrated the activation and regeneration capability of a hepatic progenitor cell population when senescence of hepatocytes was induced in an inducible MdM2-mouse model (17). In contrast, Shaub *et al.* and others failed to detect liver stem cells and demonstrated that newly formed hepatocytes all originated from pre-existing, proliferating hepatocytes (18). Although the liver is capable of regeneration, liver failure remains inevitable upon excessive tissue damage. The only curative therapy for liver failure is liver transplantation which is indicated mainly for alcoholic liver disease and hepatocellular carcinoma. However, the major limitation is the shortage of donors. Moreover, organ transplantation requires life-long immunosuppression (19). Alternatively, bio-artificial liver devices (BAL) that take over the primary functions of the hepatocytes are used to support patients on the liver transplant waiting list or to support the injured liver. The BAL have however a limited efficacy and their use is also impaired due to the limited number and the quality of available donor cells (20). Besides the urge for a renewable source of hepatocytes in regenerative medicine, the need for hepatocytes in drug development increases. Hepatocytes contain the main drug metabolizing enzymes and hepatotoxicity is one of the main reasons why drugs fail during clinical trials and are withdrawn from the pharmaceutical market. Currently, the pharmaceutical industry relies on the use of animals, chiefly rodents, to test the efficiency and toxicity of drugs. Translation to humans is not straightforward and drug metabolization differences between human and animal models remain a concern. Pharmaceutical industry is therefore continuously searching for assays that more closely mimic the human condition to predict more reliably the efficacy and toxicity of drugs.

1.1.3 Alternatives for primary human hepatocytes

Currently, primary human hepatocytes (PHH) are the gold standard for *in vitro* drug metabolization studies. However, the use of PHH is associated with some major drawbacks: (i) shortage and inter-donor variability, (ii) inability of *in vitro* expansion,

(iii) rapid *in vitro* dedifferentiation causing a loss of human specific drug transporters/enzymes and a loss of phenotypical characteristics such as polarization (21). Over the years, several attempts have been made to prevent the process of dedifferentiation. Micropatterning of PHH with supporting fibroblasts, 3D spheroid cultures and overlay cultures of PHH with two layers of extracellular matrix are some of the strategies used to retain the phenotype of isolated hepatocytes. Recently, Godoy *et al.*, demonstrated that the gene regulatory networks of cultivated PHH resemble that of inflamed livers (22). This should be considered when cultivated PHH are used for drug toxicity testing. Although several approaches could to some degree maintain the human liver metabolism, the limited availability of donors remains another major hurdle (23-25).

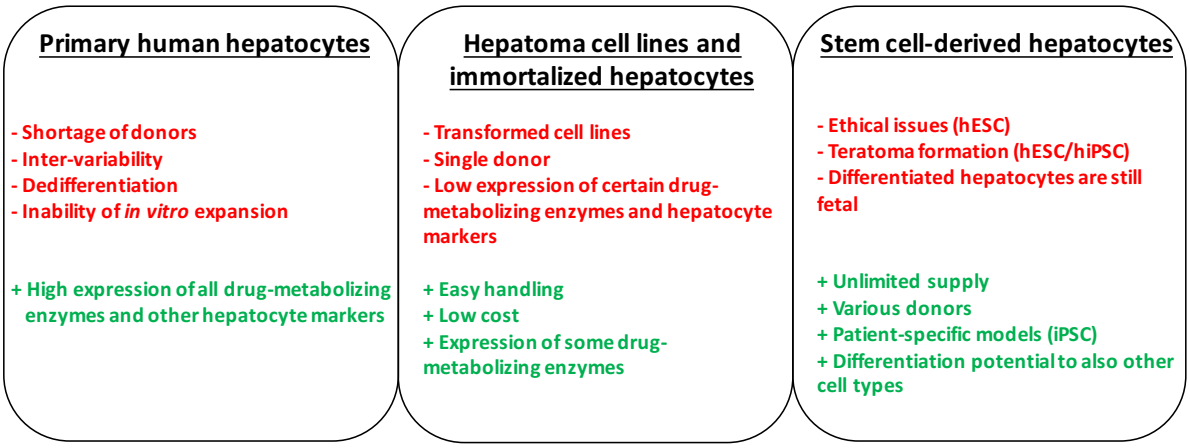

Figure 2. *Advantages and disadvantages of primary human hepatocytes and alternatives.*

Alternatives for PHH are human hepatoma cell lines and immortalized hepatocyte cell lines. Hepatoma cell lines such as HepG2 and Huh7 cells resemble some of the drug metabolizing enzymes but most of the phase I cytochrome P450 (CYP) enzymes and phase II metabolizing enzymes are only poorly expressed. Considering these limitations, their availability, unlimited growth, stability and cost are advantages for some drug metabolism and toxicity studies (26). More recently, the HepaRG cell line was derived from a hepatocellular carcinoma. HepaRG cells are bipotential proliferating cells that can differentiate to both biliary and hepatocyte-like cells upon the addition of dimethyl sulfoxide (DMSO). In contrast to other hepatoma cell lines, HepaRG cells express a higher level of certain CYP enzymes such as CYP3A4 and other drug-metabolizing enzymes. Nevertheless, HepaRG cells are derived from a single patient and remain

transformed cells that do not completely mimic the physiology of primary human hepatocytes (27).

Differentiated human stem cells are another alternative and promising renewable source of hepatocytes. Numerous protocols have been described for the differentiation of human pluripotent stem cells toward the hepatic lineage. Alternatively, certain adult stem cells have the potential to differentiate into hepatocytes. However, comparison of the different hepatocyte differentiation protocols and microarray analysis demonstrated that human stem-cell derived hepatocytes mimic better fetal than mature hepatocytes and still lack the expression of mature (drug-metabolizing) liver markers. Nevertheless, methods of hepatocyte differentiation are progressively improving holding great promise for the use of stem cell-derived hepatocytes in the future.

1.2 From stem cell to hepatocyte

1.2.1 Liver embryogenesis

Embryogenesis is a well-orchestrated stepwise process whereby totipotent stem cells are fated to either the trophectoderm or to the inner cell mass. The trophoectoderm differentiates into the extra-embryonic trophoblast that supports the developing embryo, while the cells within the inner cell mass differentiate to the primitive endoderm or to the epiblast. The primitive endoderm contributes together with the trophoblast to the extra-embryonic tissues. In contrast, the epiblast cells are pluripotent and give rise to three germ layers of the embryo -the endoderm, the mesoderm and the ectoderm- whereby the liver originates from the endoderm (28, 29). During gastrulation the epiblast cells generate the primitive streak (PS). The epiblast cells migrate through the PS to form the mesendoderm that further differentiates into the mesoderm and definitive endoderm (DE). Consensus of different studies indicate an essential role for two members of the Transformation Growth Factor Beta (TFGβ) superfamily, Activin-A and Nodal. Both factors are known to be involved in the earliest cell fate decisions, the maintenance of pluripotency and the formation of the PS/mesoderm. Besides the Nodal/Activin-A pathway, gastrulation and generation of the

PS is also regulated by other signal cascades such as canonical Wnt signaling (30-32). Throughout gastrulation, the DE is turned into the primitive gut tube that is further patterned into foregut, midgut and hindgut domains. From the ventral part of the foregut a hepatic diverticulum or foregut epithelium is formed which is adjacent to the developing heart. The liver diverticulum is lined by endoderm cells, the hepatoblasts, which invade the septum transversum (STM) of the heart to form the liver bud. The close proximity of hepatic endoderm and cardiac mesoderm is important for hepatic specification whereby the STM mesenchyme and the cardiac mesoderm secrete Bone Morphogenetic Proteins (BMPs) and Fibroblast Growth Factors (FGFs), both essential for liver formation (4, 33). Hepatoblasts are bipotential cells that differentiate into hepatocytes or cholangiocytes. The hepatoblasts continue to proliferate under the control of several growth factors, such as Hepatocyte Growth Factor (HGF), which is expressed by the STM, endothelial cells and the hepatoblasts. Hepatoblasts in contact with the portal vein form the cholangiocytes whereas hepatoblasts, not in contact with the portal vein, differentiate into mature hepatocytes (4).

1.2.2 Stem cells: a renewable source for hepatocyte generation

As briefly discussed above, stem cells hold great promise as a renewable source for the generation of hepatocytes. Both pluripotent as well as adult stem cells were shown to differentiate into functional hepatocytes *in vitro*.

Mouse and human embryonic stem cells (ESCs) were first isolated from the inner cell mass of the blastocyst in 1981 and 1998, respectively (34, 35). Embryonic stem cells are pluripotent cells capable of differentiation into the three germ layers (endoderm, mesoderm and ectoderm) and are characterized by extensive self-renewal without senescence and reconstitution of an entire organism from single cells following blastocyst injection. The unique property of differentiation into specific cell lineages holds great potential for regenerative medicine, drug development and gene therapies. Mouse and human ESCs express a number of similar markers such as *OCT4, SOX2* and *NANOG* that control pluripotency and self-renewal. However, mouse ESC express cell surface marker stage-specific embryonic antigen 1 (*SSEA1*) while human ESCs express

SSEA3 and *SSEA4*. These differences between mouse and human ESCs suggest that their downstream signaling pathways may differ (36, 37).

In 2006, Takahashi *et al* demonstrated that retroviral transfection of four transcription factors, *OCT4, SOX2, KLF4* and *c-MYC*, can reprogram mouse adult fibroblasts towards cells with ESC characteristics. They named these reprogrammed cells "induced pluripotent stem cells (iPSCs)" (38). A year later, several groups reported the successful generation of iPSCs from human somatic cells, such as human adult fibroblasts (39, 40). Although ESCs and iPSCs are highly similar, differences still exist at the molecular level. It has been recently shown that iPSCs retain an epigenetic memory of their somatic cells or origin. This implies that differentiation capacities and gene expression could be different. However, the epigenetic memory can be erased by addition of small molecules like valproic acid or longer-term passaging. By contrast, retaining the epigenetic memory might enhance the differentiation capacity of iPSC into the desired cell lineage of origin (41, 42). The ability to generate pluripotent stem cells from patients with genetic disorders is promising for *in vitro* disease modeling, disease-specific treatment testing and cell replacement therapy. Moreover, use of autologous iPSCs might circumvent the need for immunosuppression and the ethical concerns related to ESCs as they can be derived in a non-invasive way from the patient itself (43).

Alternatively, diverse adult stem cells can be isolated from the human body and differentiated into hepatocytes. Adult stem cells are undifferentiated cells that reside in differentiated tissues such as the bone marrow, brain and gut. Adult stem cells are multipotent and can differentiate into the tissue of origin or transdifferentiate into some cell types of other lineages. Human mesenchymal stem cells isolated from the bone marrow, umbilical cord blood or adipose tissue were previously shown to differentiate into hepatocytes (44, 45). Adult stem cells are characterized by extensive proliferation but do not grow indefinite such as pluripotent stem cells. They could be obtained from the patient and hence applied in an autologous manner and obviously avoid the sacrifice of the embryo. However, the hepatocyte differentiation potential is more restricted and less efficient compared to hESCs and hiPSCs (46-48).

1.2.3 Differentiation of stem cells to hepatocyte-like cells

Numerous protocols have been described to differentiate pluripotent stem cells towards hepatocytes. Pluripotent stem cells can be spontaneously differentiated in three-dimensional (3D) aggregates or embryoid bodies. Under defined conditions, these embryoid bodies spontaneously form hepatocytes (49, 50). A major drawback of spontaneous differentiation is the formation of other cell types, limiting the efficiency and yield of this differentiation approach. Alternatively, directed, two dimensional (2D), stem cell differentiation, under control of specific growth factors has proven to provide more homogeneous populations of cells with hepatocyte characteristics. In this approach, pluripotent stem cells are differentiated to the hepatic lineage by recapitulating embryonic development through the sequential addition of different signaling factors. In general, pluripotent stem cells are first differentiated into endodermal cells by the addition of Activin-A either alone or together with Wnt3A. Endodermal cells are further differentiated into hepatoblasts by bone morphogenetic protein 4 or 2 (BMP4 or 2) and fibroblast growth factor 1 or 4 (FGF1 or 4). Finally, hepatocyte maturation is induced by exposure to hepatic growth factor (HGF) often in combination with Oncostatin M (OSM) and dexamethasone (51-54).

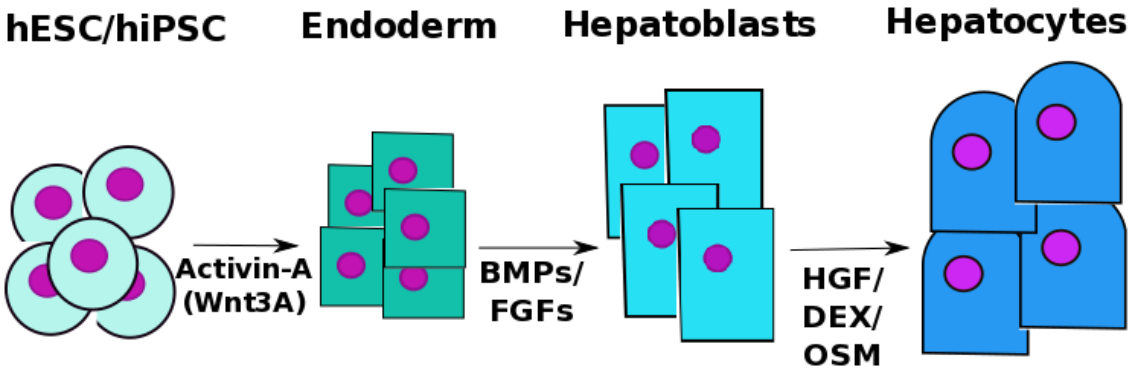

Figure 3. *Hepatocyte differentiation protocol. Consensus of different differentiation protocols. Pluripotent stem cells are differentiated to endodermal cells by Activin-A alone or together with Wnt3A. Endodermal cells are further differentiated into hepatoblasts and hepatocytes by BMPs/FGFs and HGF/Dexamethasone (DEX)/Oncostatin M (OSM), respectively.*

It is essential to review the efficiency of hepatocyte differentiation by a combination of morphological, phenotypical and functional assays. Microscopically, hepatocytes are cuboidal cells with large nuclei and abundant microvilli. In general, RT-qPCR is used for gene expression analysis to determine the commitment of stem cells to the specified lineage. Gene expression results should be confirmed by protein immunofluorescence and western blot. Hepatocytes are responsible for numerous metabolic and detoxifying functions and characterization of hepatocyte functionality is therefore a prerequisite (55). Extensive characterization of the hepatic progeny allows to compare various protocols and to review the maturity of the differentiated hepatocytes. Table 1 outlines some examples of genes, proteins and functionality assays that are generally used to define the several stages of hepatocyte differentiation.

Gene/Protein expression	Pluripotency: OCT4, Nanog, SSEA4, TRA1-60 Endoderm: MIXL1, CXCR4, EOMES, SOX17 Fetal hepatocytes: AFP, AAT, Albumin, HNF4**α** Mature hepatocytes: Albumin, AAT, HNF4**α**, NTCP, CYP3A4, CYP2D6, MRP2, UGT1A1
Functionality	Synthetic function: Albumin/AAT/AFP secretion (ELISA) Energy metabolism: Urea production, glycogen production (Periodic Acid-Shiff staining), LDL uptake Detoxification function: Phase I (CYP3A4, CYP2D6, CYP1A2) and Phase II enzymes (UGT, GST)

Table 1: *Genes, proteins and functionality assays used to review the differentiation of stem cells to hepatocytes.*

The question arises: How closely do stem cell-derived hepatocytes resemble primary human adult hepatocytes? Recently, Hanley *et al.* demonstrated that stem cell-derived hepatocytes mimic fetal rather than adult hepatocytes and are therefore often referred to as hepatocyte-like cells. The research group compared the proteome of differentiated hepatocytes to freshly isolated fetal and adult hepatocytes. Several markers such as albumin and alpha-1-antitrypsin (AAT) approximated the expression levels of adult hepatocytes. However, the expression of alpha-fetoprotein (AFP), a marker for fetal hepatocytes, was still abundant in stem cell-derived hepatocytes. In addition, several mature markers such as CYP3A4 were only expressed at low levels in the differentiated hepatic progeny cells (56). Another study compared the gene regulatory networks of

three independent hepatocyte differentiation populations to those of freshly isolated primary adult hepatocytes and cultivated hepatocytes. All the three differentiation protocols resulted in a mixed population of cells with features of not only liver but also of colon, stem cells and/or fibroblasts. The study identified unfavorable gene regulatory networks with clusters of hepatic genes that are poorly expressed in the differentiated progeny cells, clusters of highly expressed proliferation associated genes and clusters of genes that are linked to epithelial-to-mesenchymal transition and dedifferentiation of cultivated primary human hepatocytes *in vitro*. Moreover, this study demonstrated that genes that are only at low levels expressed in differentiated hepatocytes, such as the main drug metabolizing enzyme CYP3A4, overlap with genes that are downregulated during the dedifferentiation of cultured primary human hepatocytes. This observation is in line with the notion that the *in vitro* cultivation methods are currently suboptimal to induce or maintain a mature hepatocyte phenotype (57).

1.2.4 Recent advances in hepatocyte differentiation

The fetal phenotype and lack of key metabolizing/mature hepatocyte markers are currently the major limitations of stem cell-derived hepatocytes. Genome-wide characterization of the differentiated hepatic progeny cells demonstrated the lack of certain transcription factors such as pregnane X receptor (PXR), while other unfavorable transcription factors such as TWIST1 were abundantly expressed (57). Overexpression of key transcription factors is one approach to enhance hepatocyte maturation. Previous studies demonstrated that overexpression of hepatic transcription factors such as HNF4α, CCAAT/enhancer binding protein alpha (c/EBPα) and forkhead box A1 protein (FOXA1) resulted in trans-differentiation of fibroblasts into induced hepatocytes (iHEPs) (58). In addition, Nakamori *et al.*, transduced differentiated hepatocyte-like cells with activating transcription factor 5 (ATF5), c/EBPα and prospero homeobox Protein 1 (PROX1) and observed a slight improvement of some hepatocyte markers. The expression of CYP3A4 expression remained however unchanged (59). Alternatively, newly developed gene editing techniques such as CRISPR/CAS9 will allow to relatively easily knock-down and/or knock-out specific unfavorable transcription factors to study the effect on hepatocyte maturation.

Most of the published protocols differentiate pluripotent stem cells toward hepatocytes through the addition of various growth factors that mimic embryonic development (Figure 3). Small molecules are an attractive cost-effective alternative to growth factors. For instance, bromo-indirubin-3'-oxime (BIO), a glycogen synthase kinase 3 beta (GSK3β) – inhibitor that prevents the degradation of β-catenin, was previously used to replace Wnt3A during endoderm differentiation (60). In addition, small molecules can be added to induce further maturation of the differentiated hepatic progeny cells. In 2013 *Shan et al.* screened 12480 small molecules and identified two classes of molecules that either induced proliferation of primary human hepatocytes and/or improved differentiation of stem cell-derived hepatocytes. Two hits were identified that increased albumin secretion and CYP3A4 activity, while AFP secretion was decreased (61). Based on the structural properties of the identified small molecules gene regulatory networks might be unraveled to obtain more insight in the process of hepatocyte proliferation and/or maturation.

Cell-cell junctions are critical for hepatocyte maturation but are limited in 2D cultures. Therefore, several groups have cultured stem cell-derived hepatocytes in 3D spheroids to better mimic the *in vivo* microenvironment. 3D cultures show some improvement of the hepatocyte functions and induce hepatocyte polarization and bile canaliculi formation (62-65). Nevertheless, complete hepatocyte maturation was, to the best of our knowledge, not yet demonstrated. Moreover, hepatocytes reside in the liver in close contact with other liver cell types. Attempts have been made to mimic these heterotypic cell-cell interactions *in vitro* either in 2D or in 3D culture systems. Heterotypic cell-cell interactions are also crucial during liver development. To recapitulate the *in vivo* liver embryogenesis, Takebe *et al.* first differentiated pluripotent stem cells to hepatic endoderm. The differentiated hepatic endoderm was then co-cultured with human umbilical cord endothelial cells and human mesenchymal stem cells. Interestingly, the cells self-organized into 3D cell aggregates that were ectopically transplanted into immunocompromised mice. Upon transplantation, the liver buds became vascularized and improved the survival of mice with liver injury. Although the liver buds were promising, complete hepatocyte maturation was unfortunately not identified (66). Bioreactors and microfluidic devices are another promising tool to culture 2D or 3D differentiated hepatocytes cultures. In these systems oxygen tension

and the flow of nutrients, growth factors and compounds is dynamic and controllable which recapitulates better the *in vivo* liver environment (67, 68).

During the last 15 years enormous progress has been made in understanding the processes underlying hepatocyte differentiation and maturation. Despite these tremendous improvements, the generation of completely mature hepatocytes remains a major hurdle as the differentiated hepatocytes are still more closely resembling fetal hepatocytes. Nevertheless, stem cell-derived hepatocytes are a promising, more physiological, valuable alternative to hepatoma and immortalized cell lines to study drug-induced hepatotoxicity, the virology of hepatotropic viruses and liver regeneration.

1.3 Virology of hepatocyte pathogenic viruses

1.3.1 Hepatitis E virus

1.3.1.1 Genome structure and genetic characteristics

The hepatitis E virus belongs to the family of the *Hepeviridae* and is classified as the sole member of the Hepevirus genus (69). Four major genotypes of HEV are recognized. Genotype 1 and 2 are pathogenic for humans, while genotype 3 and 4 are zoonotic with reservoirs both in humans and animals including pigs (70). Recently, also genotype 5 and 6 were identified in wild boars and genotype 7 in camels (71).

HEV is a 7.2kb positive-sense single stranded RNA virus containing a capped 5' UTR and 3' UTR followed by a polyA-tail 5 (Figure 4). The RNA genome contains three partially overlapping open reading frames (ORF1, 2 and 3). ORF1 encodes the non-structural proteins, including the methyltransferase, protease, helicase and and RNA-dependent RNA polymerase (72). Various aspects of the HEV replication remain to be elucidated due to the lack of efficient and physiological relevant cell culture systems and *in vivo* animal models. Until now it remains unclear if ORF1 functions as a large polyprotein or is cleaved into its functional proteins (69). ORF2 and ORF3 partially overlap and the ORF-encoded proteins interact during replication. Their interaction is believed to be important for viral infectivity (73). ORF2 encodes the viral capsid and

contains three glycosylation sites known to be involved in the infectivity of the viral particles and the binding to susceptible cells (74, 75). A small phosphoprotein is encoded by ORF3 but the function of this phosphoprotein is still under debate. Recent findings demonstrated that ORF3 is involved in viral egress, HEV budding and virion morphogenesis (76-78).

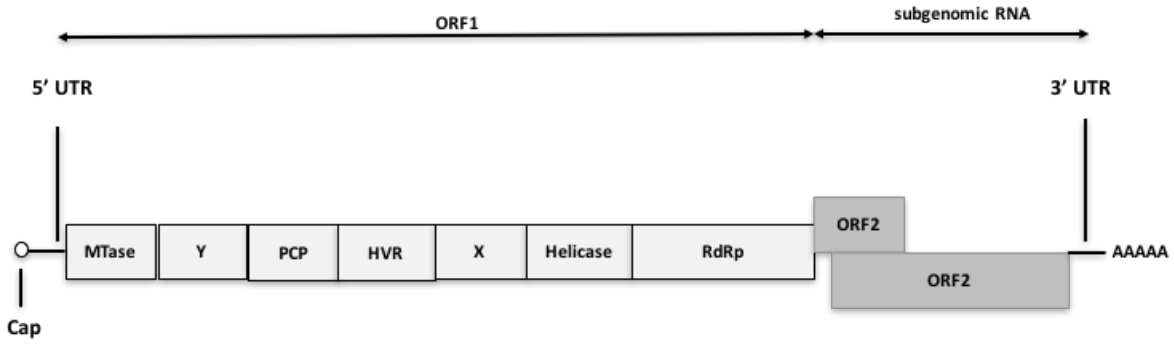

Figure 4. *HEV genome containing a capped 5' UTR and polyA-tail at the 3'UTR. Non-structural proteins (Mtase; methyltransferase, Y; Y-domain; PCP; papain-like cysteine protease, HVR; hypervariable regionX; macrodomain, RdRp; RNA-dependent RNA polymerase) are translated from ORF1. ORF2 and ORF3 are partially overlapping and encode the capsid and a small phosprotein, respectively.*

1.3.1.2 HEV replication cycle

The life cycle of the hepatitis E virus is not well understood due to the unavailability of efficient *in vitro* and *in vivo* models. The hepatitis E replication cycle seems however typical for a positive single-stranded RNA virus (Figure 5). The hepatitis E virus mainly replicates in hepatocytes (79, 80). Extrahepatic sites such as colon, spleen and lymph nodes are however also being described. Initially, the virus binds to the cells via a yet unknown receptor. Recently several groups, determined the 3D crystal structure of HEV virus-like particles (VLPs). VLPs are empty viral particles containing the N-terminal capsid protein expressed by ORF2 in Baculovirus expression system (81). Mutational analysis and attachment studies with these VLPs will aid in understanding the mechanism of HEV cell entry. Upon entry, the viral single-stranded RNA is released into the cytoplasm. Through cap-mediated translation the non-structural proteins are produced. The RNA-dependent RNA polymerase produces a negative-strand RNA replication intermediate from the positive-sense strand HEV RNA. The negative-strand

RNA is the template for the production of the full-length positive-sense RNA and the subgenomic RNA that is translated into the structural proteins of ORF2 and ORF3. Replication probably occurs in close proximity with the membranes of the endoplasmatic reticulum. ORF2 is translated into the capsid and encapsulates the newly produced viral RNA while the structural proteins encoded by ORF3 are thought to be involved in viral egress and HEV budding (82).

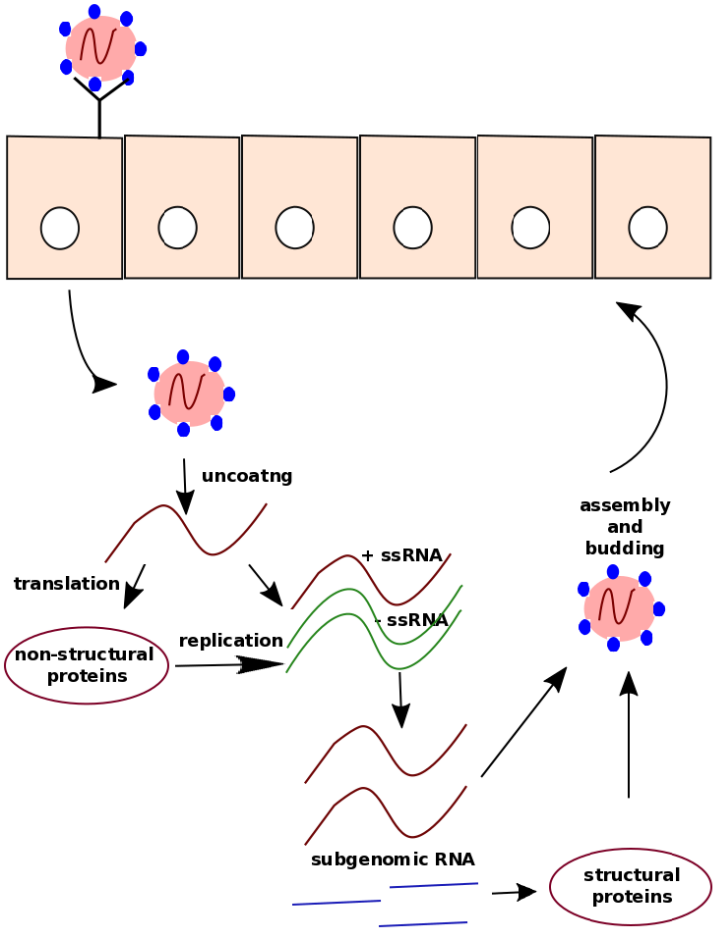

Figure 5. *HEV replication cycle. Upon HEV entry, the viral RNA is released and translated into the non-structural proteins in the cytoplasm. The RNA-dependent RNA polymerase creates the negative-strand intermediate (green) that is the template for the production of more full-length and subgenomic positive-strand RNA (red). The subgenomic positive-strand RNA is translated into the structural proteins to assemble and produce new HEV viral particles that are released from the cell.*

1.3.1.3 Epidemiology

Worldwide 20 million people are annually infected with the hepatitis E virus resulting in 3.5 million cases of acute liver injury and approximately 56000 deaths (83). Hepatitis

E is an enterically transmitted virus with the highest prevalence in Asia and Africa (84, 85). However, the incidence of hepatitis E in the Western world is increasing with a seroprevalence ranging between 5% and 50% (86-88) . Genotype 1 and 2 only infect humans and usually cause infections in developing countries through contamination of drinking water. Genotype 3 and 4 have their main reservoir in domestic pigs and are the main source of hepatitis E infection in industrialized countries due to the consumption of undercooked pig meat (69, 89). Interestingly, certain cases of fulminant hepatitis have been reported in pregnant women with mortality rates up to 30% in certain countries such as India, Iran and Ethiopia (90). The altered cellular immunity and hormonal balance are probably related to the higher incidence of fulminant hepatitis during pregnancy (91).

1.3.1.4 Pathogenesis

Hepatitis E was believed to be mainly transmitted via the fecal-oral route through drinking of contaminated water or consumption of undercooked meat in developing and industrialized countries, respectively (69, 89). However, several countries (including Canada, Japan, Germany, …) now documented that hepatitis E can as well be transmitted via blood transfusions (92-94). Usually hepatitis E is linked with acute self-limiting asymptomatic hepatitis. However symptomatic cases of acute hepatitis E have been described and are marked by flu-like symptoms such as myalgia, abdominal pain, vomiting and in some cases by clinical symptoms of acute hepatitis including jaundice, itching and increased levels of alanine aminotransferase (ALT). The incubation period of symptomatic hepatitis E is approximately 40 days (95). Although hepatitis E is mainly a causative agent of acute hepatitis, infection with hepatitis E might evolve in chronicity in immunocompromised patients. Solid-organ transplant patients as well as HIV patients have an impaired T-cell response which might lead to chronic hepatitis and eventually liver cirrhosis when left untreated. Beside chronic events, cases of fulminant hepatitis are known to occur in patients with underlying liver disease and interestingly in pregnant women (91, 96-99). As aforementioned, the reason why pregnant women are more prone to fulminant hepatitis is still unclear but might be related altered T-cell immunity. Next to hepatic manifestations, there is clear evidence that hepatitis E is linked to neurologic disorders such as Guillain-Barré syndrome and neuralgic

amyotrophy but it remains to be resolved if this is a primary or a secondary effect of hepatitis E virus infection (100, 101).

1.3.1.5 Host response and factors related to HEV

Hepatitis E is usually causing self-limiting or acute hepatitis. However, chronic infections are reported in immunocompromised patients with weakened T-cell responses. Also pregnant women have a higher incidence of fulminant hepatitis which might be related with reduced immunity during pregnancy. Studies about the immune responses to HEV are limited due to inefficient cell culture systems, the lack of small animal models and difficulties to obtain liver biopsies. An interesting study published by Prabhu *et al.* found an increase of $CD8^+$ and $CD4^+$ cells in liver biopsies of deceased patients who underwent acute HEV infection compared to healthy liver biopsies providing evidence of the induction of a cellular T-cell response upon HEV infection.

Hepatitis E virus also induces a cellular innate interferon response. The virus replicates via a double-stranded RNA intermediate that is recognized by Toll Like Receptors (TLR) and Retinoic acid-Inducible Gene I (RIG-I) like receptors upon which IFNα/β is produced. The binding of IFNα/β to the IFN receptor induces the JAK/STAT signaling cascade that results in the production of interferon stimulated genes (ISG) such as MX1 and Eukaryotic translation initiation factor 2 alpha kinase (EIF2KA2). Interferon stimulated genes can prevent viral entry, assembly, egress, translation and induce an inflammatory immune response. Nevertheless, it was demonstrated that ORF3 inhibits the phosphorylation of STAT1, thereby suppressing the induction of interferon stimulated genes and escaping the type I IFN response (102). Little is known about the interaction of host factors with the hepatitis E virus. Proteomic studies to identify host factors are usually carried out on HEV infected swine livers. The identified proteins were mostly involved in lipid and cholesterol metabolism and inflammatory and immune responses. However, these data still need to be confirmed in human (103, 104). In addition, Chandra *et al.*, demonstrated that ORF3 phosphorylates liver-enriched transcription factor HNF4α which resulted in the downregulation of several HNF4α target genes (105). Interestingly, albumin and transthyretin levels, both HNF4α target genes, were lower in the plasma of HEV infected patients compared to healthy controls (106). Due to the difficulty of HEV replication in cells/cell lines *in vitro*, most host

factors and pathways studies rely on *in vitro* replicon and overexpression systems in carcinoma cell lines to understand their role during natural human HEV infection. However, these studies have to be critically reviewed as carcinoma cell lines often have impaired host responses and overexpression systems express viral proteins at non-physiological levels.

1.3.1.6 Treatment and antiviral HEV therapy

Hepatitis E usually causes asymptomatic or self-limiting acute hepatitis in healthy individuals that does not require antiviral treatment. However, treatment is necessary for chronic hepatitis in immune suppressed transplant patients and immunocompromised patients. Initially, immunosuppressive therapy will be reduced in transplant patients to reduce the viral load. When reduction of therapy is not sufficient, antiviral therapy with ribavirin is the standard method of choice. Alternatively, pegylated interferon-α-2a can be administrated but causes severe side-effects and might lead to graft rejection. Both ribavirin and pegylated interferon-α-2a are contra-indicated in pregnant women, leaving no treatment for this group of HEV susceptible patients. Additionally, personal hygiene, quality of water supplies and adequate cooking of meat are important prophylactic measurements in the prevention of HEV infection (89, 107). Vaccination is another effective strategy to prevent HEV infection. In 2011, Hecolin® was approved by the China's State Food and Drug Administration. Hecolin® is composed of the recombinant truncated capsid protein and produces viral-like particles (VLP) that preserves the viral epitopes. The vaccine could be a potential measure to prevent outbreaks in endemic areas and as prophylactic agent in risk groups such as immunocompromised patients and pregnant women (108).

1.3.1.7 Culture systems and animal models

In vitro studies with HEV have long been hampered due to the lack of efficient cell culture systems. A breakthrough was in 2007 when Tanaka and co-workers isolated the JE03-1760F strain of genotype 3 from a fecal specimen of a Japanese patient with acute hepatitis E (109). The JE03-1760F strain was propagated efficiently in a human hepatocellular carcinoma cell line (PLC/PRF/5) and a lung cancer cell line (A549) (109).

In 2009, the same group isolated the HE-JF5/15F strain of genotype 4 from another fecal specimen of a patient with fulminant hepatitis E. This strain was as well able to efficiently replicate in the two aforementioned cell lines (110). Another strain (Kernow C1) of genotype 3 was isolated from the feces of a chronically infected HEV patient by Shukla *et al.* and was shown to efficiently replicate in HepG2/C3A cells (hepatoma cell line) (111). After various cell culture passaging the Kernow C1 strain acquired next to point mutations a ribosomal protein that probably enhances the processing and translation of the RNA (112). To further elucidate the aspects of HEV replication, infectious cDNA clones of genotype 3 (JE03-1760F and Kernow C1 strain) and genotype 1 (Sar55 strain) were constructed (112-114). Mutational analysis and cloning of these cDNA clones allowed gaining insight in the function of the different ORFs, the processing of HEV RNA and the production of HEV reporter constructs (112, 115).

Another limitation in the study of HEV replication, molecular biology and pathogenesis is the lack of a small animal model. Macaques and chimpanzees are animal models susceptible to all HEV genotypes, while pigs are a reservoir for genotypes 3 and 4 human HEV. The use of both animal models however pose ethical, financial and practical concerns. In 2016, two groups successfully infected a human hepatocyte reconstituted immunodeficient transgenic mouse model with HEV (116, 117). In this mouse model urokinase-type plasminogen activator (uPA) is under control of the mouse albumin promoter. The overexpression of uPA induces liver toxicity upon which the mouse liver can be repopulated by transplantation of human primary hepatocytes creating a humanized mouse liver. This mouse model was previously shown to be permissive for hepatitis B and hepatitis C and more recently as well for hepatitis E infection. The small animal model could now be used for drug screening. The major drawback is, however, the lack of an adaptive immune system, which makes the animal model not useful for vaccine development and the study of the immune response induced by the viral infection (116, 118).

1.3.2 Hepatitis B virus

1.3.2.1 Classification and virion structure

The hepatitis B virus belongs to the virus family of the *Hepadnaviridae* and is classified into several genotypes designed from A-J (119). The infectious 'Dane' particle has a diameter of 42nm and comprises the 3.2kb partially double-stranded relaxed circular DNA (rcDNA) within an inner nucleocapsid or core that is surrounded by an envelope that contains the HBV surface proteins. HBV is a retrovirus containing a reverse-transcriptase DNA polymerase that is linked to the 5' end of the minus strand DNA. The HBV DNA encodes four partially overlapping open reading frames that encode the surface proteins, the viral DNA polymerase, the HBV (pre)-core and protein X (HBx). The surface proteins are transcribed from the same ORF by using three different start codons. The precore protein is processed into the e antigen (HBeAg) that has no function in the viral life cycle but is a marker for the viremia in patients (120) (Figure 6). The function of the X protein remains to be resolved but there is growing evidence that the X protein is implicated in oncogenesis (121, 122) . Besides the Dane particle, two other types of particles are produced during HBV replication, which are filaments and spheres. Both these particles are not infectious as they do not contain viral DNA (123, 124).

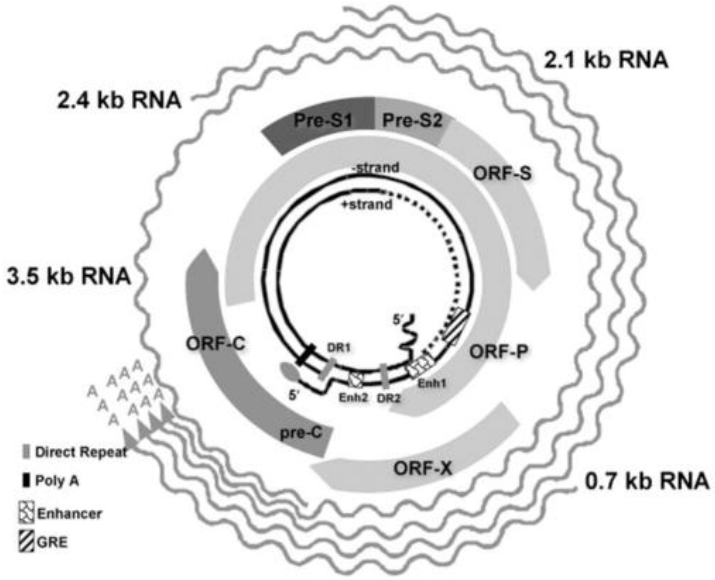

Figure 6. *HBV viral genome. The HBV genome is a relaxed-circular DNA that contains four partially overlapping open reading frames. ORF core (ORF-C) encodes the capsid, ORF S encodes the surface proteins, ORF P encodes the viral polymerase and ORF X encodes the HBV X protein. Figure is adapted from (125) .*

1.3.2.2 HBV replication cycle

HBV infection is specific for humans, chimpanzees and treeshrew and is restricted to hepatocytes. Both the small and large envelope proteins are essential for binding to the host cells. A major breakthrough in the study of HBV was in 2012 when Yan *et al.* demonstrated the binding of the preS1-domain of the large envelope protein to the bile acid transporter NTCP. NTCP appeared to be an essential receptor for HBV entry and overexpression of NTCP in non-susceptible cell lines such as HepG2 rendered them permissive for HBV infection (126). Upon entry, the capsid is released in the cytoplasm and the rcDNA translocates to the nucleus. The rcDNA is repaired by cellular DNA enzymes to form the covalently closed circular DNA (cccDNA). The cccDNA acts as the template for transcription and is a stable mini-chromosome that is extremely difficult to eradicate. Additionally, HBV DNA might integrate into the host genomic DNA, which is believed to be related to the development of hepatocellular carcinoma (HCC). The cccDNA is transcribed into four transcripts: the pre-core mRNA, the pregenomic RNA, the envelope protein mRNA and the mRNA for the X protein. The pregenomic RNA together with the viral polymerase are encapsidated within the core protein. The

polymerase reverse transcribes the pregonmic RNA into the rcDNA. After completion of the HBV rcDNA, new viral particles bud into the endoplasmatic reticulum and the golgi apparatus upon which they are secreted from the cell (Figure 7) (120).

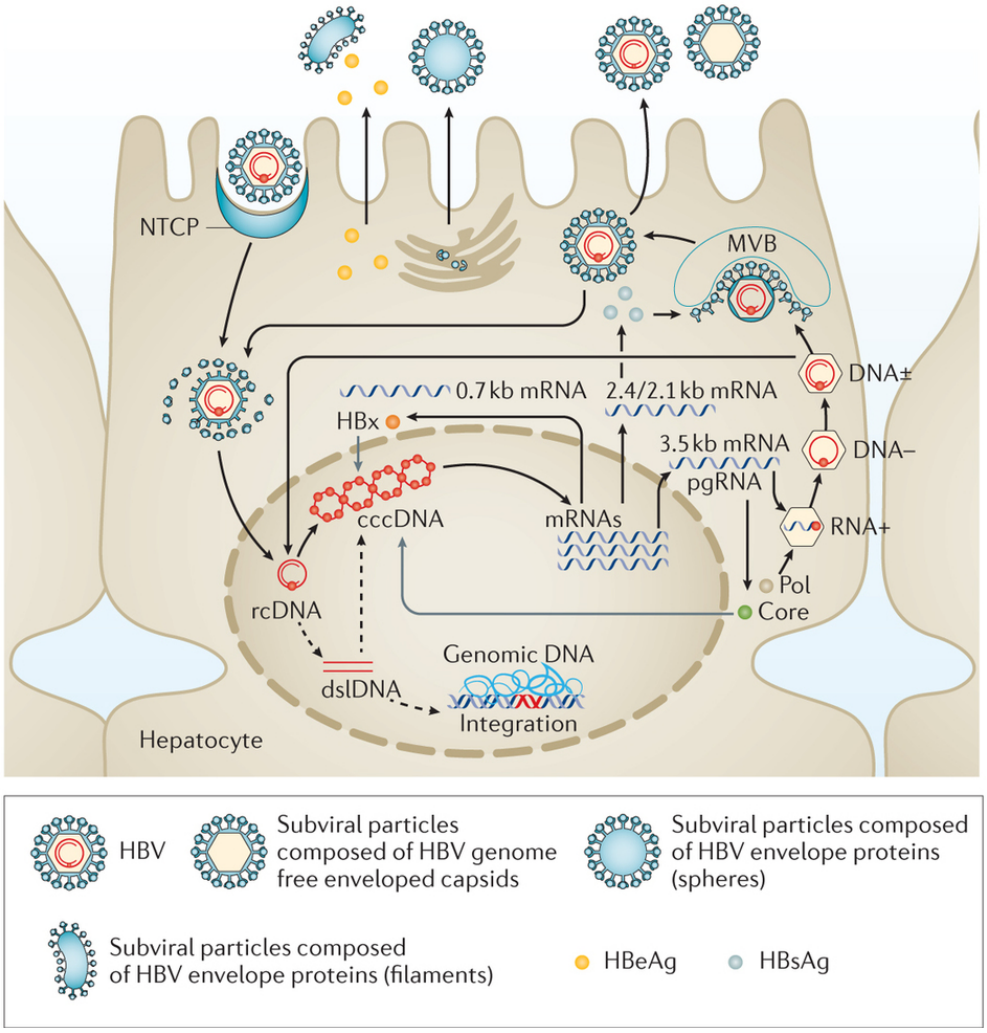

Figure 7. *HBV viral life cycle. Upon binding to NTCP, HBV enters the hepatocyte and releases the rcDNA into the cytoplasm. The rcDNA is translocated to the nucleus and repaired to form the cccDNA. The cccDNA is transcribed into the pregenomic viral RNA and other viral proteins to produce new viral particles that are secreted upon budding.* Figure adapted from (127).

1.3.2.3 Epidemiology and pathogenesis

About one third of the world population has been infected with the hepatitis B virus with over more than 350 million chronic carriers and approximately 800.000 deaths

yearly due to liver cirrhosis and hepatocellular carcinoma making hepatitis B virus still a global health care burden (128). Transmission of HBV occurs via contaminated blood or semen. In high endemic regions such as Asia, Africa and Pacific islands, HBV is mainly transmitted perinatally from mother to child, which often leads to chronic HBV infection. In areas with intermediate endemicity (Asia, central and southern Europe, Russia, South and Central America) HBV is transmitted both horizontally and through contact with infected blood or semen via sexual contact, drug abuse or contaminated needles. In low endemic areas (Northern America, western Europe, Australia, Japan) sexual transmission is the main route of infection. Prevention of HBV infection, through blood screening and a vaccination program tremendously decreased the incidence of HBV infection and hepatocellular carcinoma but the prevalence of chronic HBV infections and related HCC remains a global health concern (129).

1.3.2.4 Pathogenesis

HBV is not cytopathic and induces only a weak innate immune response. Damage to hepatocytes is therefore mainly mediated through the activation of cytotoxic T-cells in response to several viral antigens. Infections that are acquired during adulthood are mostly transient and 90% of infected adults are able to clear the virus without therapy. In contrast to adults, neonates and young children are at high risk to develop chronic infection due to a weaker adaptive immune response (120, 129). Four disease phases can be distinguished during chronic HBV infection. During the first immune tolerant phase, HBeAg is present with high levels of serum HBV DNA and normal levels of alanine aminotransferase (ALT). The immune tolerant phase can progress into the immune clearance phase with increased ALT levels and decreased serum HBV DNA. During this phase HBeAg seroconversion occurs in 10-20% of the patients and leads to a decrease in HBV DNA and a normalization of ALT levels in the inactive phase. Patients in the inactive phase have generally a good prognosis. 20-30% of patients in the inactive phase will have reactivation of HBV infection with elevated HBV DNA and ALT levels in the absence of HBeAg due to mutations in the (pre)-core. These patients are usually older and at high risk of liver cirrhosis and cancer (129, 130).

1.3.2.5 Host response and factors related to HBV

Hepatitis B is a non-cytopathic virus and not the virus itself but the immune response is the cause of liver damage. Both the innate and adaptive immune response are important for the disease progression during HBV infection. The role of the immune system is mainly studied in HBV infected chimpanzees and transgenic mouse models that express HBV antigens and HBV viral genomes. Upon infection, $CD4^+$ helper T cells produce antibodies against the viral surface proteins while $CD8^+$ cytotoxic T cells clear the infected cells and produce IFNγ and tumor necrosis factor alpha (TNFα) that will eliminate the virus without killing of the cell. The cytotoxic T cell response is crucial to eradicate the virus and impaired cytotoxic T cell responses lead to viral persistence and chronic HBV infection (131, 132). Initially, it was thought that the innate immune response was negligible as studies failed to detect a type I interferon response in animal models. However, this seems to be the consequence of the immune evasion strategies of the virus (131). Both, the HBV viral polymerase and HBx protein are known to interfere with the interferon response and abolish IFNβ production (133, 134). Moreover, HBV replicated more efficiently in IFNα/β receptor knock-out mice compared to control mice and in humans HBV infection can be treated by interferon alpha therapy (135).

Replication of HBV is tightly controlled by several cellular host factors. The HBV viral genome contains four promotor and two enhancer regions and its transcription is regulated by cellular transcription factors such as HNF4α, CCAAT/enhancer binding protein (C/EBP) and forkhead box O1 (FOXO1) (Figure 6). Most of the transcription factors that interact with the HBV genome are related to liver metabolism. Hence, it is not surprising that HBV alters the glucose and lipid metabolism of the liver (136). For instance, the HBx protein upregulates peroxisome proliferator associated receptor gamma (PPARγ) which promotes fatty acid accumulation and steatosis and in turn also enhances the transcription of HBV itself (137). Cellular host factors are also important for the formation of the cccDNA. In addition, cccDNA transcription is regulated by epigenetic modifications and is suppressed by DNA hypermethylation. Both, the formation and transcription of the cccDNA are potential drug targets that would completely eradicate the hepatitis B virus and clear the infection (138).

1.3.2.6 Prevention and antiviral treatment

Recombinant HBsAg vaccines are present since the early 1980s and have an efficiency of about 95% when administered to infants and children. Although advised by the WHO, HBV vaccination is not yet globally integrated. Other preventive measurements can be taken to reduce the risk of HBV infection such as testing of blood and organ donors, use of condoms, prevention of drug abuse and the use of sterile needles (139). The treatment of chronic hepatitis B depends on the serum HBV DNA levels, the ALT levels, liver damage and other factors such as family history and age. Two treatment options are available i.e. (pegylated-) interferon alpha treatment and nucleo(s)tide analogues (e.g. lamivudine, adefovir, tenofovir, entecavir, telbivudine). In general, treatment is recommended when HBV DNA and ALT levels are highly elevated and in patients with cirrhosis. Interferon alpha has antiviral as well as immunomodulatory effects while nucleo(s)tide analogues (NUCs) inhibit the reverse transcriptase polymerase. Because treatment does not eradicate the cccDNA in the nucleus reactivation of HBV frequently occurs after antiviral therapy. Pegylated interferon alpha has to be administrated subcutaneously once a week for 48 weeks. Pegylated interferon alpha therapy shows positive results with a high rate of HBsAg seroconversion but is associated with severe side-effects such as flu-like symptoms, fatigue and hair loss. Moreover, interferon therapy is contraindicated in patients with cirrhosis or liver decompensation. Treatment with NUCs is generally well tolerated and is administrated until HBeAg seroconversion and/or HBsAg loss. For patients with cirrhosis life-long treatment is recommended to prevent reactivation and flares of infection. The major limitation of NUCs is the problem of antiviral drug resistance, which is less with entecavir and tenofovir (128-130). Currently, a HBV entry inhibitor, Myrcludex B is examined in clinical trials. Myrcludex B is a synthetic preS1 peptide that irreversibly binds to NTCP and inactivates the HBV receptor. In addition, the peptide contains viral epitopes that might induce the production of neutralizing antibodies. In a phase I clinical trial, Myrcludex B was well tolerated and potential clinical efficacy is suggested from a phase II clinical trial (140).

1.3.2.7 Culture systems and animal models

Many aspects of the HBV viral life cycle have been revealed in hepatoma cell lines. However, infection of hepatoma cell lines was mostly inefficient or even impossible. Initially, HBV DNA genomes and plasmids encoding viral proteins were transfected or transduced in hepatoma cell lines to examine the host immune response, viral replication, produce HBV inocula and to identify antiviral drugs (127, 141). The HepaRG cell line, obtained from a tumor from an HCV-infected patient, was the first hepatoma cell line that supported the complete HBV replication cycle, including entry and production of cccDNA. HepaRG cells are marked by bipotency and can differentiate to both biliary and hepatocyte-like cells (142). Entry in hepatoma cell lines was the main limiting factor for infection of these cell lines. Since the discovery of NTCP as a crucial entry receptor, cell lines were made that stably overexpress NTCP. The HepG2-NTCP cell line was shown to be an efficient cell culture system that supported the complete replication cycle of HBV (126). This model will further facilitate the *in vitro* study of the hepatitis B virus. Although hepatoma cell lines are an indispensable *in vitro* HBV culture model, they remain transformed, abnormal cell lines with deficits in host responses. The gold standard for the study of hepatotropic viruses remains human primary hepatocytes, which are readily infected. Alternatively, stem cell-derived hepatocytes could be used and partially overcome the limitations of primary hepatocytes scarcity and inter-donor variability (127, 141). Although the phenotype of stem cell-derided hepatocytes is still not fully mature, they are susceptible to many hepatotropic viruses such as HCV, HBV and HEV and are more physiologically relevant to study host factors and antiviral drugs compared to hepatoma cell lines (143-145).

Hepatitis B has a narrow host tropism and only infects human and chimpanzees which impeded the use of small animal models for *in vivo* studies. Initially, mouse models were used that overexpressed HBV viral proteins or the full-length HBV genome to screen antivirals and to study the immune response upon HBV infection. However, these mouse models have some major limitations. As the HBV genome is integrated, the viral HBV DNA cannot be completely eradicated and certain steps of the viral life cycle such as entry are lacking. Humanized mice models, such as the uPA/SCID mice are another alternative. In humanized mice the complete replication cycle of HBV can be examined in an in vivo setting. However, these mice are immunodeficient which limits

the use to study the host response upon infection (127, 141). Several groups are therefore trying to develop mouse models with both a humanized liver and immune system by transplanting both human hepatocytes and human hematopoietic stem cells (146, 147).

1.3.3 ZIKA virus

1.3.3.1 History and virology

Zika virus (ZIKV) was first isolated in 1947 in a rhesus monkey in Uganda (148). Some years later, the virus was isolated in humans and the first outbreaks of Zika virus were reported in regions of Africa. Until the 1980s, Zika virus infection was believed to be limited to African and Asian countries, in which different strains of the Zika virus occur. It was not until 2007 that the first outbreak outside of Africa/Asia was reported in Micronesia, part of Oceania (149). In 2013, there was another endemic in French Polynesia, which affected about 11% of the total population (150). Initially, Zika virus was mostly asymptomatic or related with mild symptoms and was not believed to cause a major threat to the public health. However, in 2015, the first cases of Zika virus infection in Brazil and neighboring countries in Latin America were reported evolving into a new outbreak outside of Africa or Asia, with an estimated 1.5 million cases of Zika virus infection (Figure 8). Zika virus infections in Latin America were thought to be related to the Asian strain and were associated with an increase in the incidence of microcephaly in newborns causing severe birth defects (151, 152).

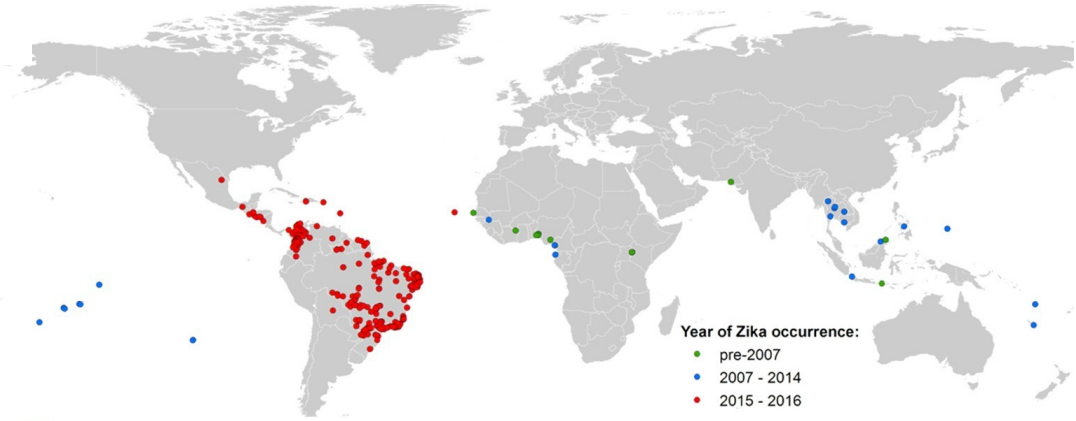

Figure 8. *Global distribution map of the Zika virus. Zika virus was first reported in Africa and Asia and later on spread to regions of Oceania and South America. Figure adapted from (153)*

The Zika virus is an arthropod borne virus and is transmitted by *Aedes* mosquitos. In addition, there is evidence that the virus can be transmitted from humans to humans, perinataly, by sexual contact or by blood transfusion (154, 155). The virus belongs to the family of the *Flaviviridae* and is related to the yellow fever and dengue virus. Zika virus is an encapsulated positive single-stranded RNA virus with a genome of approximately 11 kb. The viral RNA encodes three structural and seven non-structural proteins and is thought to replicate in a similar way as other flaviviruses (152).

1.3.3.2 Clinical manifestations

Zika virus infection is often asymptomatic or causes flu-like symptoms such as headache, fever, malaise and myalgia. However, during the French Polynesian and Latin American outbreak, there was an increase of Guilllain-Barré syndrome cases. Guillain-Barré syndrome is an immune-mediated peripheral neuropathy that can be triggered by bacterial or viral infections and leads to muscle weakness (151). During the Latin American outbreak Zika infection was for the first time linked to congenital abnormalities and microcephaly. Newborns with microcephaly have decreased brain development and abnormally small heads. In 2015, during the Brazilian outbreak, the number of newborns with microcephaly increased 20 times. In addition, viral RNA has been detected in the amniotic fluid of infants born with microcephaly and congenital defects where often documented when pregnant women underwent symptomatic Zika virus infection (151, 156, 157). Currently, there are no treatment options nor vaccines

available for Zika virus infection. Prevention of infection and control of the *Aedes* mosquito are therefore the only and necessary measurements, especially for pregnant women (151, 152).

1.3.3.3 Culture systems and in vivo models

Until the large endemic outbreak in Latin America, Zika virus was an understudied pathogen due to the mostly benign nature of infection. However, the first viral Zika strain was already isolated (MR 766) in 1947 from a rhesus monkey in Uganda (148). Later on, additional strains were isolated from patients both from the African and the Asian lineage. Vero cells (African green kidney monkey cells) are commonly used to propagate flaviviruses such as dengue virus and were also proven useful for Zika viral stock production. However, the major limitation is the interferon deficiency of Vero cells (158). Successful infection of human keratinocytes and dendritic cells already unraveled some of the tissue specificity of the Zika virus (159). Recently, several studies demonstrated that the Zika virus infects hiPSC-derived neural progenitor cells and induces cytopathogenic effects (160-163).

Small animal models have also provided additional evidence of Zika virus replication in both neuroprogenitors and neurons. However, only mouse models with a deficit in interferon immunity were susceptible for Zika infection. After infection, high viral load was detected in many tissues, including the brain, blood, testes, spleen and liver. Wild-type mice were however not susceptible for Zika infection hampering the study of immune-mediated pathogenesis (164-166). Li et al., further demonstrated that Zika virus infected more efficiently neuroprogenitor cells than neurons in developing mouse embryos, which supports the observation of microcephaly and neurological abnormalities in human newborns (167). Both the *in vitro* cell culture models as well as the small animal models should aid in the understanding of the pathology of Zika virus infection and the production of effective drugs and vaccines.

Chapter II: Objectives

The study of the biology of the replication of hepatotropic viruses and inhibition thereof is hampered by the lack of cell culture models that resemble the *in vivo* cellular environment required by these viruses. During the last years, many groups, including the Verfaillie lab, developed and optimized differentiation protocols for the production of hepatocyte-like cells (52, 54, 168, 169). Despite the major progress made, the yield and purity, as well as the functional maturity of the stem cell-derived hepatocytes still need to be further increased. The main aim of this thesis was to further optimize the differentiation protocol of human hESC/hiPSC, to create a human cell culture model that is capable of propagating hepatotropic viruses.

(1) Infection of stem cell-derived hepatocytes with the HEV

The objective aimed to determine if hESC – and hiPSC-derived hepatocyte-like cells could support the *in vitro* infection and replication of the hepatitis E virus. We aimed to demonstrate the susceptibility of hPSC – hepatocyte-like cells to HEV by means of RT-qPCR, RNA fluorescence in-situ hybridization and to assess inhibition of viral replication and to test if infected cells produced infectious virions, using a re-infection assay.

(2) Determine if HEV infection is hepatocyte-specific

As knowledge about HEV tissue tropism is limited, we aimed to examine if HEV infection and replication occurs in hepatocytes only. The use of PSCs allows generation of other cell types, aside from hepatocyte-like cells. We exploited this hPSC characteristic, to determine if HEV also infects and replicates in neuroprogenitor and mesodermal cells.

(3) Evaluate the effect of DMSO on hepatic differentiation/maturation

DMSO has been reported to enhance the *in vitro* differentiation of stem cell-derived hepatocyte-like cells and to reduce dedifferentiation of primary human hepatocytes. We assessed the effect of different concentrations of DMSO on definitive endoderm formation as well as on maturation of hepatocyte-like cells from hESCs and hiPSCs, and

aimed to determine the possible mechanism(s) underlying the effect of DMSO on hepatocyte differentiation from PSCs.

(4) Infection of the stem cell-derived hepatocytes with the HBV

We hypothesized that DMSO treated hPSC-hepatocyte-like cells might be infected by the hepatitis B virus (HBV). In an initial proof of principle experiment, we tested if DMSO treated hPSC-HLCs could be infected with the HBV and used immunofluorescence to demonstrate whether or not hPSC-HLCs were infected with HBV.

(5) Infection of stem cell-derived hepatocytes with the ZIKV

Not much is known about the tissue tropism of Zika viral (ZIKV) replication. ZIKV belongs to family of the *Flaviviridae,* and other members of this virus family such as the dengue virus and yellow fever virus are known to infect and replicate in hepatocytes. Because of the analogy between these viruses, the objective was to examine if hPSC-HLCs support ZIKV replication. We also compared infection of hPSC-HLCs and hepatoma cells to determine if hepatocyte-like cells would be superior for assessing ZIKV infection and drug sensitivity.

Chapter III: Stem cell-derived hepatocytes: a novel model for hepatitis E virus replication

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3.1 Introduction

In 1978, a novel non-A, non-B hepatitis virus was discovered which was identified in 1983 as the hepatitis E virus (HEV). HEV is an important and emerging cause of acute self-limiting hepatitis (170, 171). However, fulminant cases of hepatitis may occur particular in pregnant women with mortality rates up to 20%-30%. In immunocompromised solid-organ transplant recipients and HIV-infected patients the virus may result in chronic hepatitis, which evolves, in some patients, rapidly to cirrhosis, graft loss and death (99, 172-176).

In vitro HEV culture systems have only recently been established. Therefore, relatively little information is available on the biology of HEV infection and replication, for example the mechanism by which HEV enters the host cell remains elusive. It is believed that following entry in the cell, HEV replicates in the cytoplasm through a negative strand RNA ((-)ssRNA) intermediate synthesized by the viral RNA-dependent RNA polymerase (RdRp) (82). Although HEV is a hepatotropic virus, there is evidence that it may also replicate in extrahepatic sites; for instance, HEV RNA has been detected in cerebrospinal fluid, possibly linking HEV infection to neurological conditions that are occasionally observed in patients with (chronic) HEV infections (100, 101).

In recent years, HEV strains have been isolated from fecal specimens of patients with fulminant (JE03-1760F strain) and chronic hepatitis (Kernow-C1 strain) that replicated efficiently in cell culture (111, 112, 115). Cell lines that are used to study the molecular biology of HEV are the hepatoma cell lines Huh7, HepG2/C3A, PLC/PRF/5, HepaRG and surprisingly the lung-derived A549 adenocarcinoma cell line (111, 112, 115, 177). These transformed cell lines are physiologically less relevant to study HEV replication than primary hepatocytes, the latter are however not readily available. Hepatoma cell lines in general poorly express drug metabolizing enzymes and might lack certain host factors that are important to study infection with hepatotropic viruses. For instance, hepatoma cell lines can only be infected with the hepatitis B virus when they overexpress the sodium taurocholate co-transporting polypeptide (126, 178-180). Although human primary hepatocytes would be the best cell source to study HEV, they are short in supply.

Human embryonic (hESC)- and induced pluripotent stem cell (hiPSC)-derived hepatocytes are a valuable alternative to primary hepatocytes. Compared to primary hepatocytes, hESC and hiPSC have numerous advantages, including their capacity to self-renew long-term without loss of differentiation potential, their potential to differentiate to any given cell type and their ability to generate patient-specific disease models (181-184). Although stem cell-derived hepatocytes mimic fetal but not adult hepatocytes (56), numerous studies have demonstrated the use of differentiated hepatocytes to study drug-induced liver toxicity (25-28). Moreover, hiPSC-derived hepatocytes make it possible to model liver diseases *in vitro* and to assess patient-specific drug responses (185-188). hESC- and hiPSC-derived hepatocyte-like cells (hPSC-hepatocytes) may as well be valuable cell culture models to study the infection of hepatocytes with hepatotropic viruses and parasites (189). Others and we demonstrated earlier that hESC/hiPSC- hepatocytes can be infected with the hepatitis C virus (143, 190, 191) and that such cultures offer a model to study virus-host interactions (192). Furthermore, PSC-hepatocytes have been shown to be susceptible to infection with the hepatitis B virus (145).

We here demonstrate that PSC-hepatocytes support the complete HEV replication cycle, infection, replication and generation of infectious virions, making this an attractive and relevant *in vitro* model system (in non-cancerous hepatocytes) to study the biology of HEV replication that and to aid in the design of therapeutic strategies against the virus. Because PSC can not only differentiate into hepatocytes, but also to mesodermal and neuroprogenitors (NPCs), this PSC derived model also allowed to demonstrate that non-endodermal germ-line progeny do not allow HEV entry, even if they do support HEV replication upon transfection with a subgenomic HEV replicon.

3.2 Materials and methods

3.2.1 Virus. Wild-type and 1634R infectious HEV stocks (Kernow-C1 p6, genotype 3, GenBank accession number JQ679013) (112) were derived from plasmid DNA as described (193, 194).

3.2.2 Virus inoculation. Day 20 PSC-derived hepatocyte progeny was infected with 300 µL HEV stock diluted to 3 x 10^7 viral RNA copies/mL per well and incubated for 24h at 37°C in a 5% CO_2 humidified incubator. After 24h, the inoculum was removed and cells were washed 5 times with 500 µL of DMEM, before addition of 500 µL of hepatocyte differentiation medium. Medium was changed every other day by collecting 300 µL and replacing it with 350 µL fresh hepatocyte differentiation medium. Infection of mesodermal and neural progenitor cell differentiations was performed similarly. Infection experiments were ended 12 days post infection. Supernatant was collected every other day during medium changes while cells were lysed with 350 µL RLT buffer (Qiagen, Hilden) 4, 8, 10 and 12 days post infection. An identical protocol was used for a clinical plasma sample from an acutely HEV genotype 3-infected patient (a kind gift from Heiner Wedemeyer, Hannover, Germany). The plasma sample was diluted 1:3 in hepatocyte differentiation medium and subsequently used for inoculation.

3.2.3 Cell cultures. The fibroblast cell line BJ1 was previously reprogrammed to hiPSC by lentiviral transduction of OCT4, c-MYC, KLF4 and SOX2 (39). The hESC line H9 (WA09) was purchased from WiCell Research Institute (Madison, WI). Both hiPSC and hESC were maintained on inactivated mouse embryonic fibroblasts (iMEF) in hESC medium (DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% KnockOut Serum Replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 1 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/mL bFGF (basic fibroblast growth factor) (Peprotech, Rocky Hill, NJ) in a humidified 5% CO_2 incubator at 37°C. HepG2/C3A cells (a kind gift from Luc Verschaeve, Scientific Institute of Public Health, Brussels, Belgium) and Huh7 cells (a kind gift from Ralf Bartenschlager, University of Heidelberg, Germany) were cultured in

Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in a humidified 5% CO_2 incubator at 37°C.

3.2.4 Hepatocyte differentiation. hESC and hiPSC were differentiated towards hepatocytes following a previously described protocol with minor modifications (169) (Figure 9). Briefly, cells were detached from iMEF with 0.05% trypsin and plated at ± 8.75 x 10^4 cells/cm^2 on matrigel (BD Biosciences, San Jose, CA) coated wells. When cells reached 70-80% confluency, hepatocyte differentiation was induced by the sequential addition of different growth factors as described (169), except that Activin-A was used at 50 ng/mL instead of at 100 ng/mL. All growth factors were purchased from Peprotech.

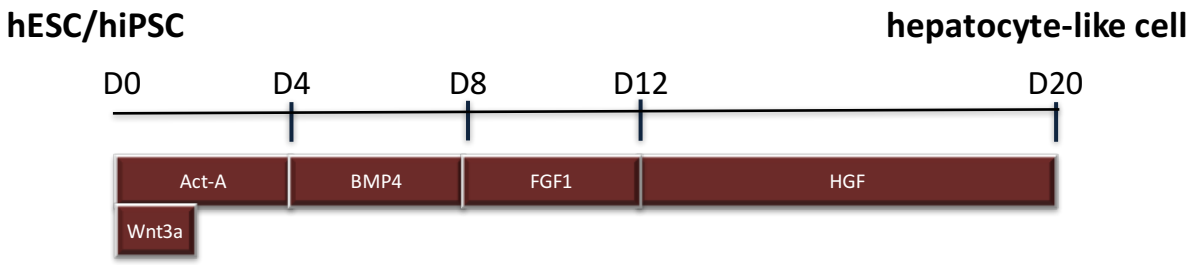

Figure 9. *Hepatocyte differentiation protocol.*

3.2.5 Mesoderm differentiation. To differentiate hESC towards mesodermal cells, cells were harvested from iMEF with 0.05% trypsin and plated at ± 8.75 x 10^4 cells/cm^2 on matrigel-coated wells. Using a published protocol (195), with modifications, cells were differentiated by the sequential addition of bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 1 (FGF1). Mesodermal cells were stained with 1 µg/mL PE mouse anti-human platelet-derived growth factor receptor (PDGFRβ) antibody (BD Pharmingen, San Diego, CA) or 1 µg/mL PE mouse IgG2a, κ isotype, as control (BD Pharmingen). Mesoderm differentiated cells were defined as being more than 95% positive for PDGFRβ by flow cytometry analysis using a FACS-Canto (BD) (195).

3.2.6 Neuroprogenitor differentiation. hESC (H9) were harvested from iMEF and seeded at a density of 5 x 10^5 cells/cm^2 on matrigel-coated plates in mTeSR1

medium (BD) containing 10 µM ROCK inhibitor. When they reached 100% confluency, medium was changed to neural induction medium (NIM) (Neural Maintenance Medium (Neurobasal medium, B27 supplement, Glutamax, N2 supplement, 2-mercaptoethanol, MEM NEAA, sodium pyruvate (all purchased from Life Technologies, Waltham, MA), Pen/Strep and Insulin (purchased from Sigma-Aldrich) (NMM) supplemented with 10 µM SB431542 (Sigma-Aldrich) and 1 µM dorsomorphin (Tocris, Bristol)) and replaced every day until day 12 as previously described (196). From day 12 onwards, cells were passaged with dispase and replated onto poly-L-ornithine and laminin-coated plates in NIM at a density of 2 x 10^5 cells/cm^2. The day after, medium was changed to NMM supplemented with 20 ng/mL FGF2. Neural progenitor cells were passaged when they reached confluency and were cryopreserved as single cells between day 30 and 35 of differentiation.

3.2.7 RNA extraction and quantitative reverse-transcription PCR (qRT-PCR). For gene expression analysis, RNA was isolated from differentiated progeny cells by the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following manufacturer's procedures. Genomic DNA was eliminated using the On-Column DNase I Digestion kit (Sigma-Aldrich). The Superscript III First-Strand synthesis system (Invitrogen) was used for subsequent cDNA synthesis. qPCR was performed with the Platinum SYBR green qPCR supermix-UDG kit (Invitrogen) in a ViiA 7 Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for normalization. Sequences of qRT-PCR primers are listed in Table 2. Viral RNA was isolated from the supernatant using the NucleoSpin RNA virus kit (Macherey-Nagel, Düren) according to the manufacturer's instructions. Intracellular RNA from infected stem cell progeny was extracted using the RNeasy Mini Kit (Qiagen). qRT-PCR for viral RNA was performed as previously described (194). Viral RNA detected in lysates was normalized to total RNA content as measured by spectroscopy (Nanodrop ND-1000, Thermo Fischer Scientific).

Gene	Forward	Reverse
GAPDH	TCAAGAAGGTGGTGAAGCAGG	ACCAGGAAATGAGCTTGACAAA
AFP	TGAGCACTGTTGCAGAGGAG	GTGGTCAGTTTGCAGCATTC
ALB	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA
AAT	AGGGCCTGAAGCTAGTGGAT	TCCTCGGTGTCCTTGACTTC
DLX2	ACGCTCCCTATGGAACCAGTT	TCCGAATTTCAGGCTCAAGGT
PAX6	GTGTCTACCAACCAATTCCACAAC	CCCAACATGGAGCCAGATG
BLBP	GGACTCTCAGCACATTCAAGAA	CCACATCACCAAAAGTAAGGGT
EIF2AK2	GAGAATTTCCAGAAGGTGAAGGT	ATTCCCCATGGATAATCCTTCT
MX1	TGCTTATCCGTTAGCCGTGG	CGCCAGCTCATGTGCATCT
ISG15	GAGAGGCAGCGAACTCATCT	CTTCAGCTCTGACACCGACA
IFNβ	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG
ssHEV1	TGAAAATGACTTTTCGGAGTTTG	ATGTTATTCATTCTACCCG
ssHEV2	ACACCGTTTGGAACATGGC	AACATGCCAATAAGGTTATG
ssHEVTag	CGGTCATGGTGGCGAATAATGAAA ATGACTTTTCGGAGTTTG	CGGTCATGGTGGCGAATAAATGTTAT TCATTCTACCCG

Table 2. *Primer list used for RT-qPCR experiments*

3.2.8 Strand-specific PCR detection of HEV RNA. Specific amplification of positive- and negative-sense HEV RNA was performed as described, with slight modifications to primers and procedure (197). For first-strand synthesis, 1 µL of RNA extract was mixed with 1 µL of 10 µM primer solution (ssHEVTagF1 for negative-strand assay, ssHEVTagR1 for positive-strand assay) and diluted with H_2O to a final volume of 11.75 µL. Samples were heated at 65°C for 5 min and incubated on ice. To each sample, 1.25 µL of 10 mM dNTP mixture, 4 µL of 5x first strand buffer (Life Technologies), 1 µL of 0.1 M DTT, 1 µL of RNasin RNase-inhibitor (equals 40 units, Promega, Madison, WI) and 1 µL of SuperScript III reverse transcriptase (Life Technologies) was added. Reaction was incubated at 55°C for 1h and consequently inactivated at 70°C for 15 min. Next, 50 units of ExoI (NEB, Ipswich) were added and reaction was incubated at 37°C for 30 min. DNA was purified with Qiagen PCR purification kit by the manufacturer's instructions with final elution in 30 µL of buffer EB. Five µL of the eluate was used in a 25 µL PCR reaction with GoTaq HotStart MasterMix (Promega) and primer pairs Tag – ssHEVR1 for negative-strand assay or ssHEVF1 – Tag for positive-strand assay with following temperature scheme: 2 min at 95°C, 40 cycles of 30s at 95°C, 30s at 55°C and 1 min at 72°C, and finally 5 min at 72°C. DNA was purified with the Qiagen PCR purification kit and 1 µL was used in a second PCR reaction with primer pair ssHEVF2 – ssHEVR2 with following temperature scheme: 2 min at 95°C, 20 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C, and finally 5 min at 72°C. Amplified fragments were separated on a 2% agarose gel (expected fragment length: 415 bp). For validation, RNA

extracts derived from culture medium and lysates of infected and uninfected HepG2/C3A cells were used. Contrary to the findings of Chatterjee et al, we did not observe the presence of negative-strand RNA in viral particles (i.e. in the culture medium) (197).

3.2.9 Immunofluorescence. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min. at room temperature (RT), permeabilized with 0.2% Triton X-100, blocked with 5% normal donkey serum (Jackson Laboratory, Bar Harbor, ME) and stained overnight at 4°C with primary antibodies and isotype controls (Table 3). Afterwards, cells were incubated with appropriate secondary antibodies together with Hoechst (Sigma-Aldrich) for 60 minutes at RT (Images were taken by the AxioimagerZ.1 fluorescence microscope.

Antibody	Catalog number	Company	Dilution
OCT4	Sc-8628	Santa Cruz	1:200
SSEA4	Sc-21702	Santa Cruz	1:500
NANOG	AF1997	R&D	1:400
A1AT	A0012	Dako	1:2000
HNF4α	Ab41898	Abcam	1:200
ALB	A0001	Dako	1:4000
AFP	A008	Dako	1:2700
NESTIN	MMS-570P	Covance	1:200
PAX6	PRB-278P	Covacs	1:200
αSMA	C6198	Sigma	1:200

Table 3. *Antibody list used for immunofluorescence staining.*

3.2.10 Albumin and AAT ELISA. Enzyme-linked immunosorbent assay (ELISA) for albumin and AAT was performed according to the manufacturer's procedure (Bethyl, Montgomery, TX). Briefly, at day 20 of the hepatocyte differentiation protocol, supernatant was collected and incubated with a primary albumin or AAT antibody for 60 min. at RT. Afterwards an HRP-detection antibody was added for 60 min. followed by TMB-peroxidase solution for 15 min. at RT. The reaction was stopped by a 0.18M H_2SO_4 solution and the OD was measured on a microplate reader at a wavelength of 450 nm. A standard curve was used to quantify the amount of albumin and AAT secretion.

3.2.11 Intracellular flow cytometry. For intracellular AAT flow cytometry staining, a single cell suspension was made by liberase treatment (Roche, Basel, Switzerland) followed by fixation with 4% PFA for 15 min. at RT. Next, cells were permeabilized with 0.1% saponin and blocked with 10% goat serum (Dako, Glostrup, Denmark) for 45 min. at RT. Afterwards cells were stained with 0.0625µg/200µL/10^6 cells anti-AAT antibody (Dako) or a rabbit IgG isotype control (BD Pharmingen) for 1h at RT. After 1h, a secondary Alexa Fluor 647 antibody (1:1500) (Invitrogen) was used for 30 min. at RT. Cells were analyzed by flow cytometry analysis using a FACS-Canto (BD).

3.2.12 Viral infection inhibition experiments. Ribavirin (ICN Pharmaceuticals, Quebec) and interferon alpha 2b (Intron-A®, Schering-Plough, Kenilworth, NJ) were used to inhibit viral replication. Ribavirin (100 µM) and interferon (1000 U/mL) were added to the differentiation medium starting at the time of the inoculation until the end of the infection experiment.

3.2.4 Re-infection assays. HepG2/C3A cells were seeded into 6-well plates at 2 x 10^5 cells per well and incubated for 24h at 37°C. Day 8, 10 and day 12 culture medium samples from HEV-infected stem cell-derived hepatocytes (a fixed volume of 400 µL for each sample) were diluted with 500 µL of DMEM supplemented with 10% FBS and inoculated on HepG2/C3A cultures. Infection was allowed to proceed for 4h at 35°C. Afterwards, inoculum was removed, cell layers were washed three times with 2 mL of phosphate-buffered saline, 2.5 mL of DMEM with 10% FBS and 1% pen/strep was added and cultures were incubated at 35°C for 20 days with regular changing of the medium as described (37). After 20 days, cellular lysates were prepared as described.

3.2.5 QuantiGene ViewRNA fluorescence in-situ hybridization (FISH). RNA FISH was performed using the QuantiGene ViewRNA protocol. Briefly, infected or uninfected stem cell-derived hepatocytes or HepG2/C3A cells were fixed with 4% formaldehyde for 30 min at room temperature. After fixation, cells were permeabilized with detergent solution for 5 min (Affymetrix, Santa Clara, CA) and treated with proteinase K (Affymetrix) for 10 min. Cells were hybridized for 3h at 40°C with a

Quantigene ViewRNA designed probe covering the region 858-1791 of ORF1 of the HEV clone Kernow-C1 p6 (Accession number HQ389543). After hybridization the signal was amplified by sequential reaction of the PreAmplifier and the Amplifier mix (Affymetrix) followed by conjugation with the fluorescent dye-conjugated label probe (Affymetrix). Cells were counterstained with DAPI (Affymetrix). Images were taken by the AxioImagerZ.1 fluorescence microscope.

3.2.6 HEV replicon replication. Genotype 3 reporter replicon viral RNA was derived from a plasmid encoding Kernow-C1 p6/luc, (kind gift from Suzanne U. Emerson) (13). Viral RNA was *in vitro* transcribed from *Mlu*I-linearized plasmid DNA with the RiboMAX Large Scale RNA Production System-T7 (Promega) and capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). Huh7 cells were seeded into 24-well plates at 4 x 10^4 cells per well, mesoderm cells and neuroprogenitors were seeded at a density of 2 x 10^5 cells per 24 well. Cells were transfected with capped RNA transcripts (200 ng per well) 24 hours later using Lipofectin (Life Technologies) according to the manufacturer's instructions. Transfected cells were incubated at 37°C and 30 µL of cell culture medium was removed from each well and stored at -80°C every day. After 3 days, media were thawed and *Gaussia* luciferase activity was measured in 20 µL culture medium with the Renilla luciferase assay system (Promega). For mesodermal cell differentiations, luminescence signal was normalized for the approximate number of seeded cells where necessary.

3.2.7 Statistics. Data values represent average ± standard error of the mean (SEM) and were analyzed by the two-tailed Student's t test. p-values < 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****) were considered statistically significant.

3.3 Results

3.3.1 Stem cell-derived hepatocyte cultures support the full HEV replication cycle

The pluripotency of hESC (H9) and hiPSC (BJ1) lines was first confirmed (Figure 10).

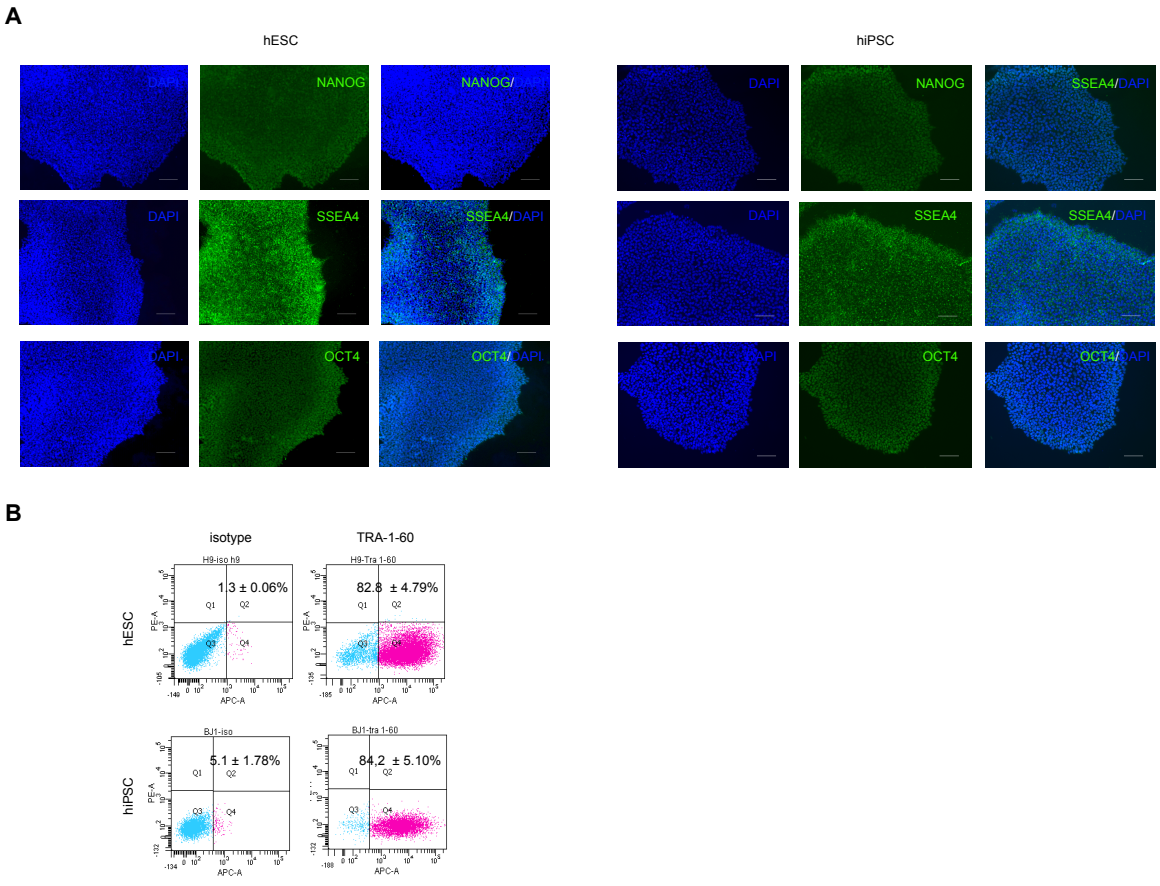

Figure 10. *Characterization of pluripotent hESC and hiPSC. (A) hESC (H9) and hiPSC (BJ1-iPSC) were stained for pluripotency markers NANOG, SSEA4 and OCT4 (Scale bar = 100 µm). Images are representative of three independent experiments. (B) Flow cytometry analysis for TRA-1-60 demonstrated that both hESC and hiPSC lines were >80% for TRA-1-60. Results represent the mean of three independent experiments ± SEM*

PSCs were differentiated towards hepatocyte-like cells (hPSC-hepatocytes) during a 20-day differentiation protocol. Immunofluorescence staining, flow cytometry and gene

expression analysis demonstrated that PSC-hepatocytes were positive for AFP, HNF4α, AAT and albumin, and secreted albumin and AAT (Figure 11).

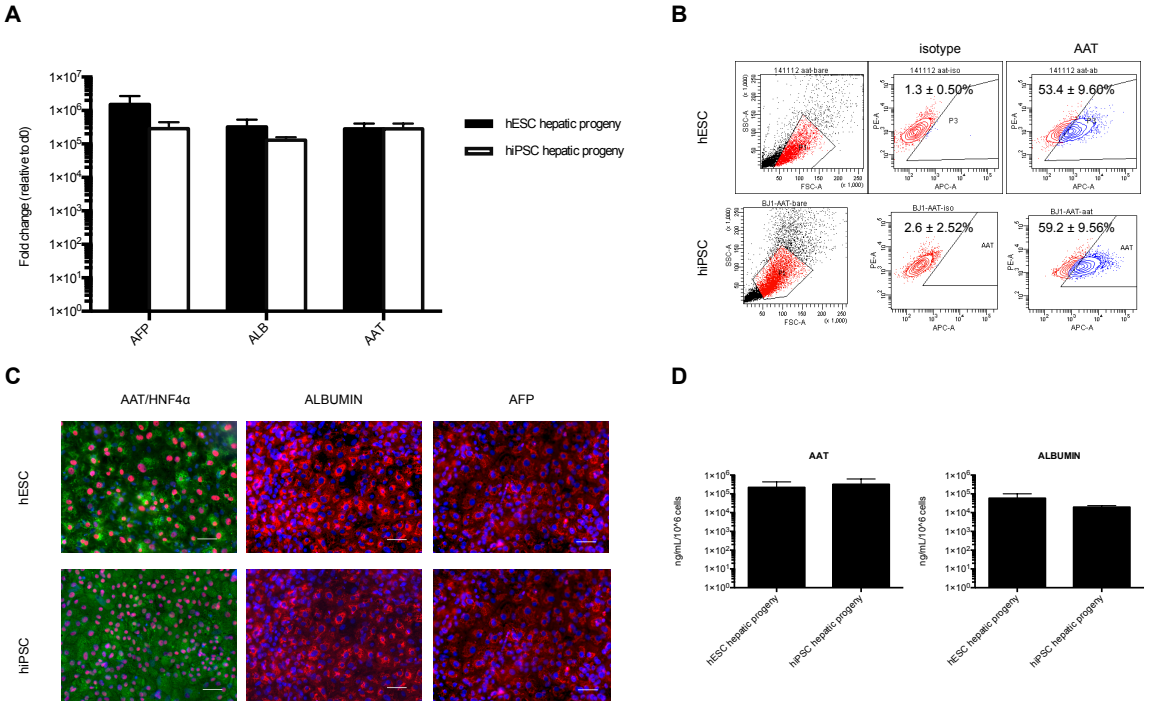

Figure 11. *Characterization of hPSC-derived hepatocytes. (A) Expression of hepatocyte markers (AFP, ALB and AAT) in hESC and hiPSC hepatic progeny cells. Results represent the mean of three independent experiments ± SEM. (B) Intracellular flow cytometry analysis for AAT demonstrated that approximately 50% of the hESC and hiPSC hepatocyte progeny were positive for AAT. Results represent the mean of three independent experiments ± SEM. (C) Immunofluorescence staining for AAT, HNF4α, albumin and AFP showed abundant expression of the hepatocyte proteins in both hESC- and hiPSC-derived hepatocytes (Scale bar = 100 µm). Images are representative of three independent experiments. (D) ELISA for AAT and albumin on day 20 of the hepatocyte differentiation protocol. Both hESC- and hiPSC-derived secrete comparable amounts of AAT and albumin while there was no detectable level of AAT or albumin secretion for hESC and hiPSC (data not shown). Results represent the mean of four independent experiments ± SEM.*

PSC-hepatocytes were inoculated with Kernow-C1 p6 genotype 3 HEV that carries a G1634R mutation in the C-terminal region of the HEV polymerase. We recently reported that this mutation increases the replication capacity of genotype 3 HEV *in vitro* (193). HEV RNA was detected (by qRT-PCR) in the culture medium of infected hESC-

hepatocytes on day 2 after infection and increased overtime from 1.5 ± 0.4 x 10^5 to 5.1 ± 1 x 10^5 viral RNA copies ml^{-1} on day 12 after infection (Figure 12A). It should be noted that the copy numbers were not corrected for culture medium replacement. Indeed, every other day, half of the culture medium was replaced, which accounts for a final dilution factor of about 40. Viral RNA was also detected on day 4, 8, 10 and 12 after infection in lysates of the infected hESC-hepatocytes and the viral RNA copy number remained constant during this time-span (Figure 12A). The number of RNA copies in the supernatant of hiPSC-hepatocytes increased from 6 ± 2 x 10^4 to 2.9 ± 0.5 x 10^5 RNA copies ml^{-1} between day 2 and 12 after infection, and remained stable at the intracellular level (Figure 12A). Although the differentiation capacity of hESC and hiPSC was comparable (Figure 11), intracellular HEV RNA copy numbers in hiPSC-hepatocytes were significantly lower (Figure 12A). This might be explained by the presence of a stronger innate immune response in hiPSC-hepatocytes. Indeed, we detected a higher level of transcripts for the interferon response genes, EIF2AK2, MX1, ISG15 and IFNβ in hiPSC-hepatocytes compared with hESC-hepatocytes (10 days post infection) (Figure 12B).

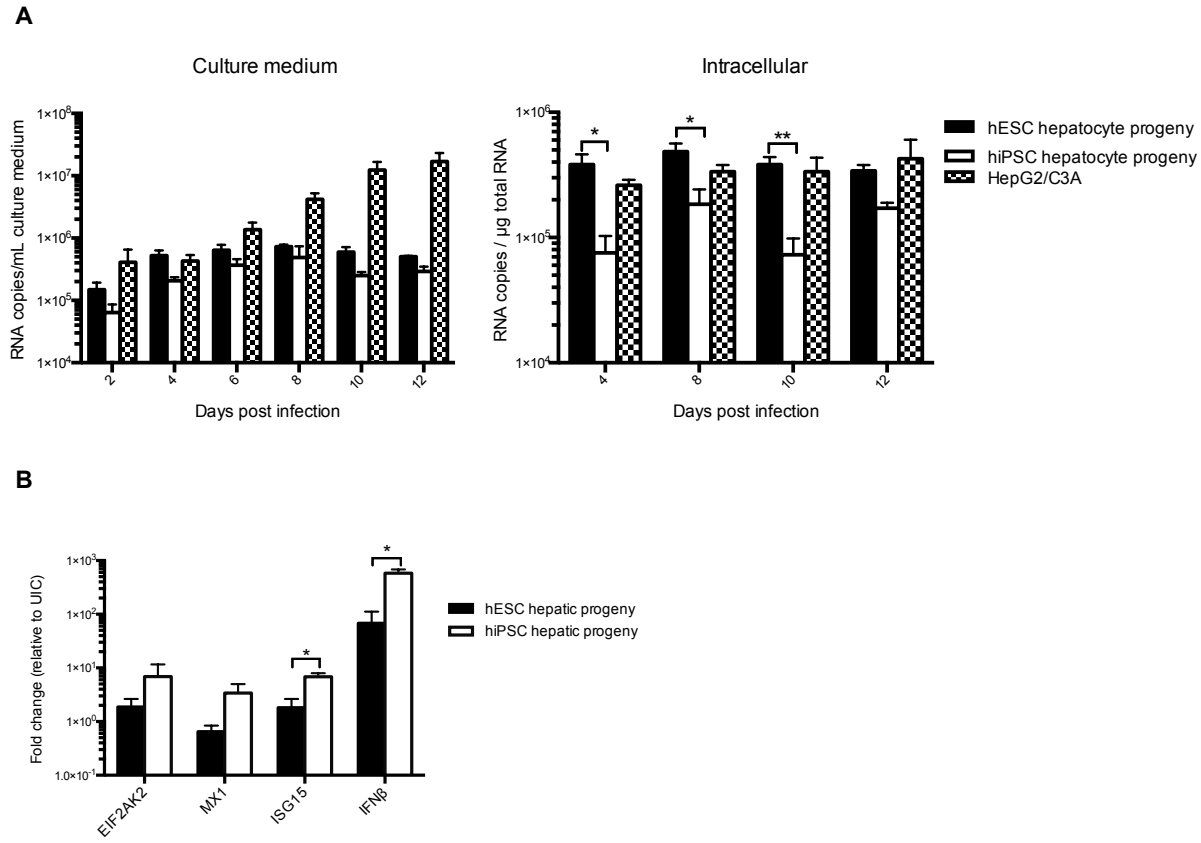

Figure 12. *HEV infection of hESC- and hiPSC-derived hepatocyte cultures. (A) After inoculation of day 20 hESC (n=6), hiPSC (n=3)-hepatocytes and HepG2/C3A cells (n=3), HEV RNA was detected in the culture supernatant on the indicated time points and quantified by qRT-PCR. HEV RNA was quantified in intracellular lysates 4, 8, 10 and 12 days after infection. Results were expressed as the mean of six (hESC), three (hiPSC) and three (hepG2/C3A) independent experiments ± SEM. (B) qRT-PCR results 10 days after infection for innate immune response genes demonstrated higher expression of interferon response genes in hiPSC-hepatocytes compared to hESC-hepatocytes. Results represent the mean of three independent experiments ± SEM relative to uninfected control cells (UIC).*

As control, hPSC-hepatocytes were inoculated with a UV-inactivated virus stock. No HEV RNA copies were detected intracellularly or in the culture medium of the infected hPSC-hepatocytes (data not shown). The characteristics of the infection of hPSC-hepatocytes by HEV was directly compared with that of the established HEV model in HepG2/C3A cells (112). HepG2/C3A cells were infected under the same conditions as hPSC-hepatocytes (Figure 12A). At the intracellular level there was only a slight difference between the viral RNA levels detected in infected hPSC-derived hepatocytes and the infected HepG2/C3A cells. Between day 2 and day 6 post infection, HEV RNA

copy numbers in supernatants of HepG2/C3A cells were comparable with those in PSC-hepatocyte supernatants. However, the number of RNA copies continued to increase in supernatants of infected HepG2/C3A cells whereas levels reached a plateau in infected hPSC-hepatocytes from day 4-6 onwards. This might be explained by the continued proliferation of HepG2/C3A cells during the infection process whereas hPSC-hepatocytes are mostly non-dividing. Alternatively, the fact that the Kernow-C1 p6 strain has been adapted to the specific environment of HepG2/C3A cells through 6 consecutive passages might explain this difference (112).

Finally, we inoculated hPSC-hepatocytes with serum from an acutely infected genotype 3 HEV patient. However, no robust HEV replication was observed, which is not unexpected, since *in vitro* culturing of clinical isolates of HEV has proven to be extremely difficult, even in primary hepatocytes (198, 199).

3.3.2 Both (+)ss and (-)ss HEV RNA is detected in the infected hepatic progeny

Next RNA *in-situ* hybridization was used to localize (+)ss viral RNA in hPSC-hepatocytes. This technique specifically detects the presence of HEV RNA by hybridization of an RNA probe to the positive-sense viral RNA. Small clusters of positive cells were detected 10 days post infection in infected hPSC-hepatocytes, but not in mock-infected control cells or infected cultures that had been stained without the HEV probe (Figure 13A).

Because HEV replicates, akin to other (+)ssRNA viruses, through a (-)ssRNA intermediate, presence of negative-strand RNA is the ultimate proof of active viral replication (47). To this end, we used a strand-specific RT-PCR protocol. No (+)ssRNA or (-)ssRNA was detected in non-infected lysates and culture medium collected from mock-infected cells (Figure 13B). (+)ssRNA was detected in the lysates and the supernatant of HEV-infected hESC- and hiPSC-hepatocytes whereas (-)ssRNA was only detected in the lysates, but not in the supernatant of infected hESC- and hiPSC-hepatocytes. This confirms that the hPSC-hepatocytes support HEV replication (Figure 13B.)

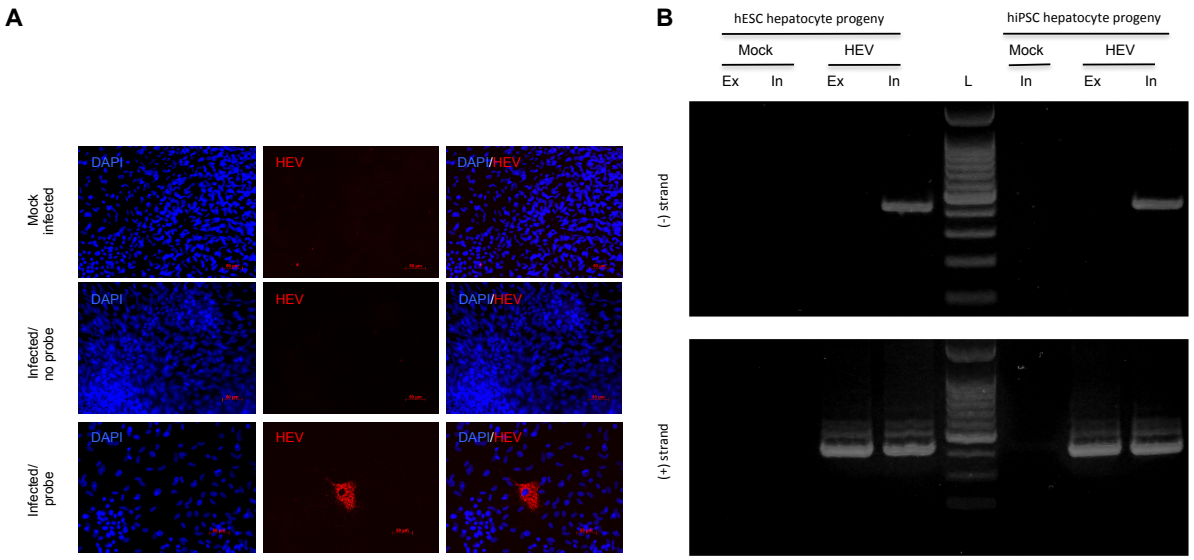

Figure 13. *Detection of both (+)ss and (-)ss HEV RNA in hepatocyte progeny. (A) Ten days after infecting ESC-derived hepatocytes, cells were fixed and stained for HEV RNA by in-situ RNA hybridization. Mock-infected and infected cells stained without HEV RNA probe were used as negative controls. Images are representative of three independent experiments (Scale bar = 50 µm). (B) Strand-specific RT-PCR demonstrated that negative-strand HEV RNA was detected in the intracellular lysates of ESC- and iPSC-hepatocytes while positive-sense RNA was detected in all inoculated cultures in both culture medium samples and lysates. Images are representative of three independent experiments. Ex, RNA extracted from culture medium samples; In, RNA extracted from lysates; L, 100 basepair DNA ladder (Promega).*

3.3.3 Inhibition of HEV replication

To further corroborate the observation that HEV replicates in hPSC-derived hepatocyte progeny, the effect of the replication inhibitors ribavirin (RBV) and interferon alpha (IFN) was studied (194). Treatment of HEV-infected hepatocyte progeny with 100 µM ribavirin or 1000 U/mL IFN markedly and significantly reduced HEV RNA copies number both intra- and extracellularly in infected hESC- (Figure 14A) and hiPSC-hepatocytes (Figure 14B). The observed lag time in the antiviral effects of IFN and RBV in hPSC-hepatocytes at day 4 post infection could be due to the detection of residual viral RNA remaining from the inoculum that has attached to the cell, but has not been

internalized. By day 8 post infection, these remnants are probably degraded or released, explaining the strong antiviral effect observed at this point.

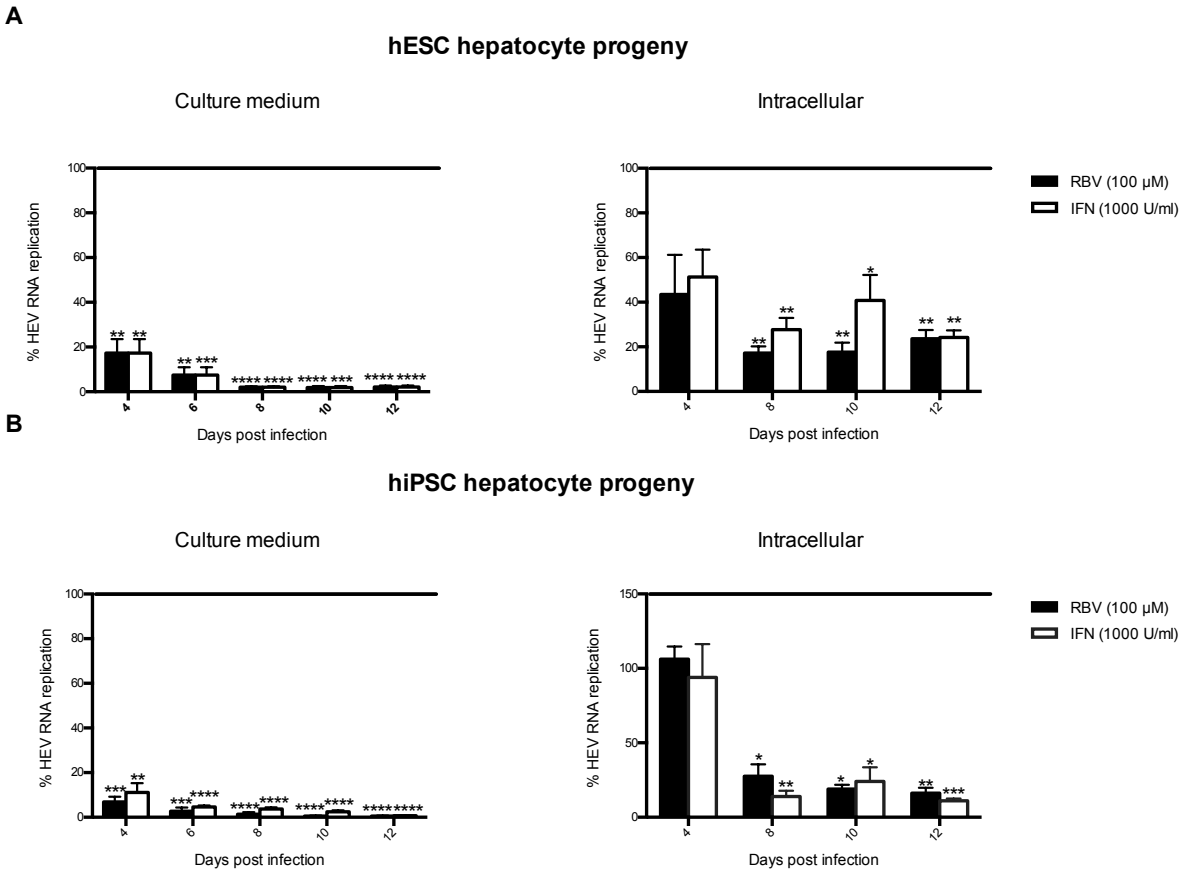

Figure 14. *Inhibition of HEV replication. (A) HEV replication was inhibited hESC-hepatocytes cells by 100 µM ribavirin (RBV) and 1000 U/mL interferon alpha (IFN) added from the inoculation until the end of the infection experiment. (B) Similarly, infected h hiPSC-hepatocytes treated with 100 µM RBV and 1000 U/mL IFN displayed strongly inhibited HEV replication as analyzed by qRT-PCR. The percentage HEV replication represents the number of RNA copies in the treated condition relative to the number in the untreated condition. Results represent the mean of three independent experiments ± SEM.*

3.3.4 Comparison of the replication efficiency of wild-type and 1634R mutant HEV in hESC-derived hepatocyte progeny

We recently demonstrated that a G1634R mutation in the HEV polymerase enhances the *in vitro* HEV replication in HepG2/C3A and Huh7 cells (193). Because of this replication advantage, initial studies were performed with HEV genotype 3 containing

the 1634R mutation. In line with these results, the HEV RNA copy number in hPSC-hepatocytes or supernatant following infection with wild-type HEV was consistently lower than in cultures infected with mutant HEV, although this did not reach statistical significance (Figure 15).

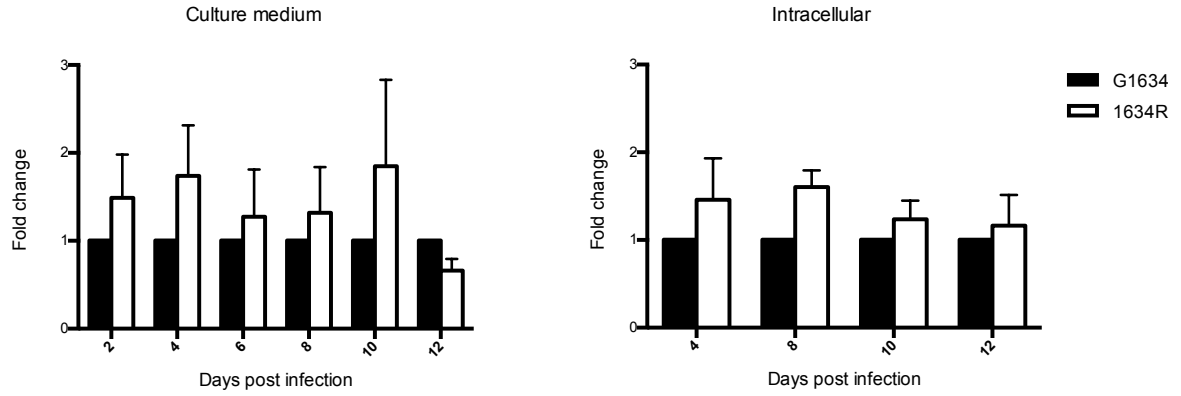

Figure 15. *Wild-type versus mutant HEV replication. Hepatocyte differentiated cells were infected with either wild-type (G1634) or mutant (1634R) HEV. Mutant HEV has a slight replication advantage compared to wild-type as quantified by qRT-PCR. Results represent the mean of three independent experiments ± SEM.*

3.3.5 hESC-derived hepatocyte progeny infected with 1634R mutant HEV produces infectious virus capable of re-infecting hepatoma cells

Detection of infectious virus in culture supernatant of infected hPSC-hepatocytes provides evidence that these cells support the full replication cycle of HEV. Additional confirmation was obtained by assessing whether HEV infected PSC-hepatocyte supernatant contained infectious virions. When day 8, 10 or 12 culture medium of wild-type HEV-infected hESC-hepatocytes (3 independent experiments) was used for reinfection of HepG2/C3A, in only one of the 9 samples detectable levels of intracellular HEV RNA were measured. By contrast, culture supernatant collected from medium of hESC-hepatocytes that had been infected with mutant R1634 HEV, resulted in infection in 4 of 9 HepG2/C3A cultures (data not shown). The moderate number of successful re-infections may be explained by the rather low viral titer in the culture medium combined with the relatively low efficiency of infection of HepG2/C3A cells. Nevertheless, the infectivity of the culture medium collected from mutant 1634R HEV-

infected hESC-hepatocytes provides further evidence that these cells support the complete HEV replication cycle, including the production of infectious viral particles.

3.3.6 hPSC-derived mesoderm and neuroprogenitor cells do not support complete HEV replication

To assess whether HEV infection and replication occurs specifically in hPSC-derived hepatocytes and not in other cell types, hESC-derived mesodermal cells (hESC-mesoderm) and differentiating NPCs (hESC-NPCs) were generated. Flow cytometry analysis of the hESC-mesoderm demonstrated that >95% of cells stained positive for PDGFRβ, a marker expressed by mesenchymal cells (Figure 16A). qRT-PCR analysis a 10-100 fold increase in levels of mesoderm specific transcripts (alpha smooth muscle actin (αSMA), leech homeobox 1 (LOX1) and collagen type 1 alpha 1 (COL1A1) (Figure 16B). Immunofluorescence staining confirmed the presence of αSMA while there was no detectable expression of AAT, HNF4α, albumin or AFP (Figure 16C). qRT-PCR analysis of the hESC-NPCs demonstrated a 10-1000 fold increase in transcripts for NPC-specific transcripts (distal-less homeobox 2 (DLX2), PAX6 (paired box 6) and brain lipid-binding protein (BLBP)) (Figure 16D). In addition, hESC-NPCs stained positive for nestin and PAX6 but negative for AAT, HNF4α, albumin or AFP (Figure 16E).

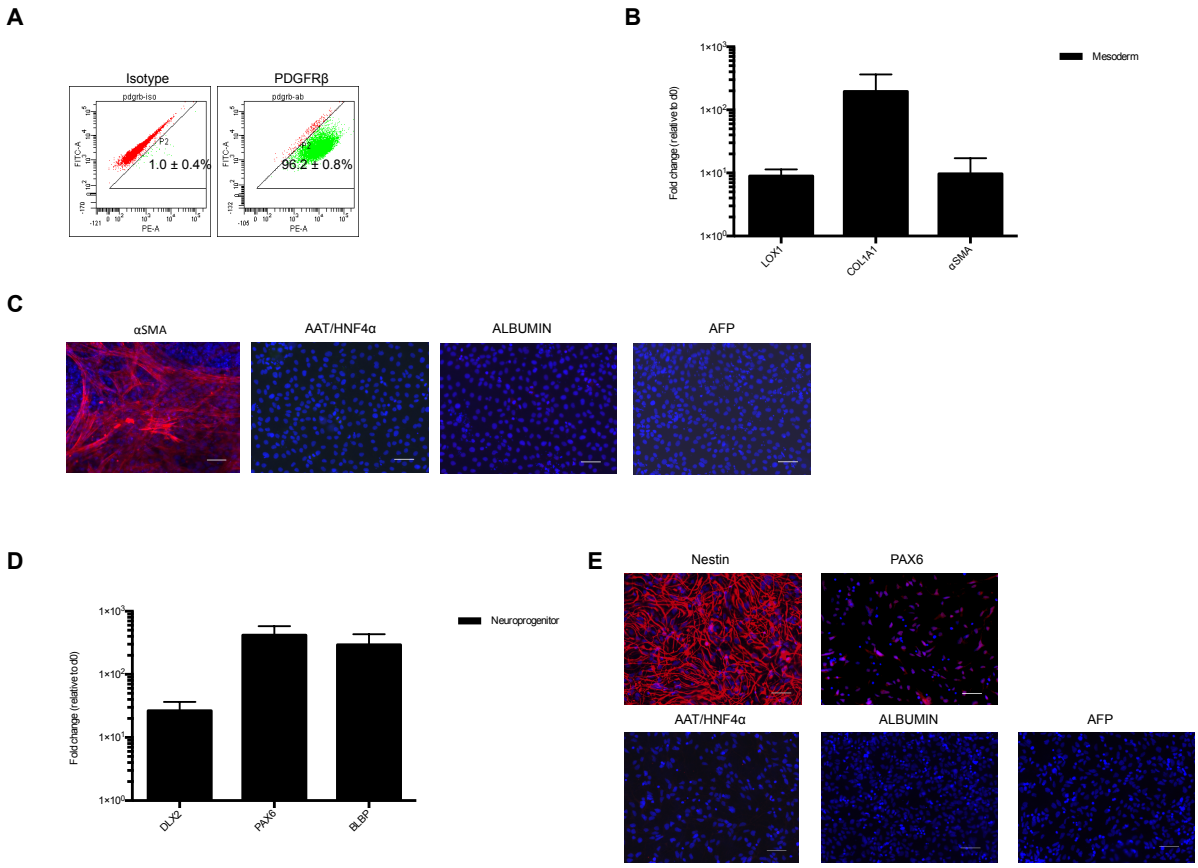

Figure 16. *Characterization of mesoderm and neuroprogenitor cels. (A) Mesoderm cells were >90% positive for PDGFRβ as analyzed by flow cytometry. Results represent the mean of three independent experiments ± SEM. (B) The expression of mesoderm genes (LOX1, COL1A1, αSMA) was determined by RT-qPCR. Results represent the mean of three independent experiments ± SEM. (C) The expression of αSMA was further confirmed by immunofluorescence staining while mesoderm cells stained negative for hepatocyte markers (AAT/HNF4α, ALBUMIN, AFP) (Scale bar = 100 µm). Images are representative of three independent experiments. (D) The expression of neuroprogenitor genes (DLX2, PAX6, BLBP) was determined by RT-qPCR. Results represent the mean of three independent experiments ± SEM. (E) Neuroprogenitors stained positive for NESTIN and PAX6 but negative for AAT/HNF4α, ALBUMIN, AFP (Scale bar = 100 µm). Images are representative of three independent experiments.*

hESC-mesoderm and -NPCs were infected under the same conditions that were used to infect hPSC-hepatocytes. On day 2 post infection, viral HEV RNA was detectable in the culture supernatant of hESC-mesoderm and -NPCs, however from day 4 onwards the number of RNA copies in both cultures decreased significantly, which was in contrast to the situation in culture medium of hESC-hepatocytes (Figure 17). Similarly, HEV RNA

copy numbers in cell extracts of hESC-mesoderm and -NPCs decreased progressively (Figure 17).

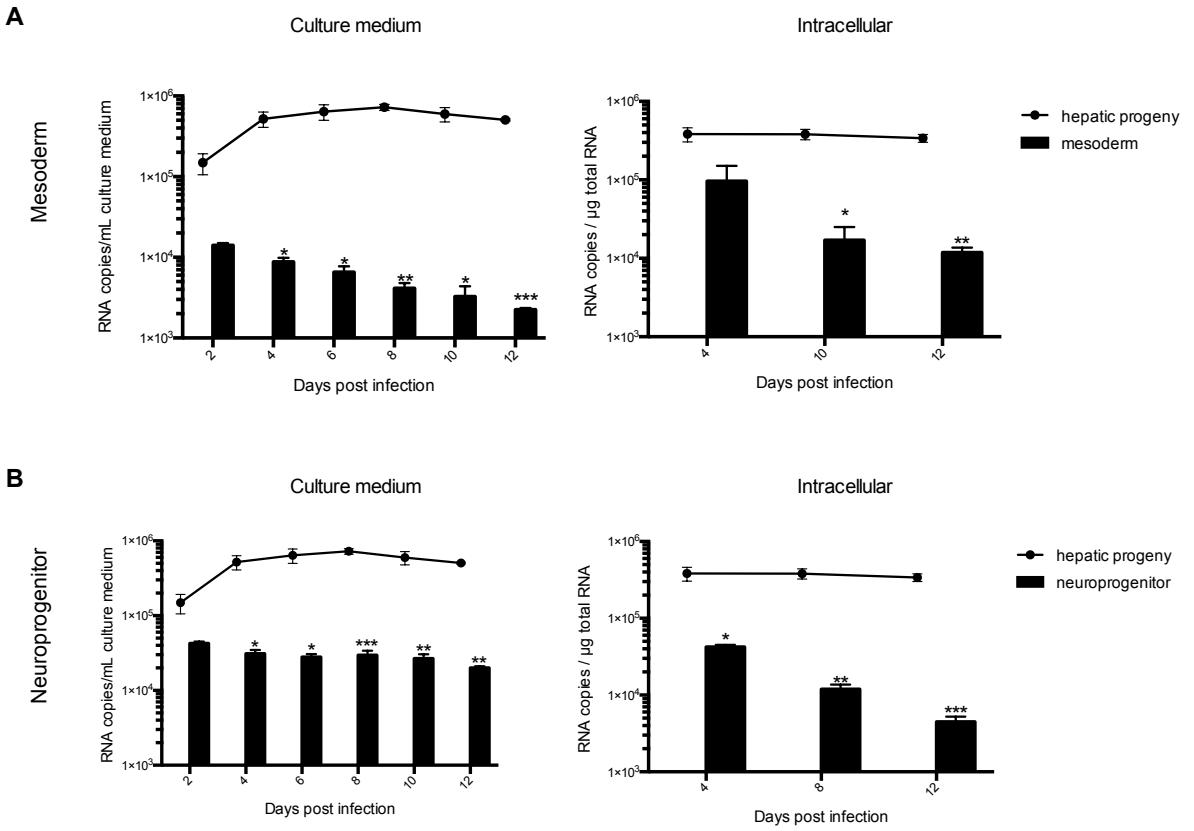

Figure 17. *HEV specifically infects stem cell-derived hepatocytes. (A&B) Viral HEV RNA was detected in the culture medium and intracellular lysates hPSC-mesoderm and NPCs on the indicated time points by qRT-PCR. Results were expressed as the mean of two (mesoderm) and three (NPC) independent experiments ± SEM. Also shown (line above the bar graphs), is the level of HEV RNA copies in the supernatant of hPSC-hepatocytes and in the cellular lysate of hPSC-hepatocytes, as reference.*

Neither RBV nor IFN had a significant effect on the viral RNA levels, suggesting that the viral RNA detected was not the result of HEV replication but rather a remnant of the inoculum (Figure 18A). Moreover, no negative strand RNA was detected in the lysates of infected hESC-mesoderm and -NPCs, confirming that these non-endodermal lineages do not support replication of the HEV (Figure 18B).

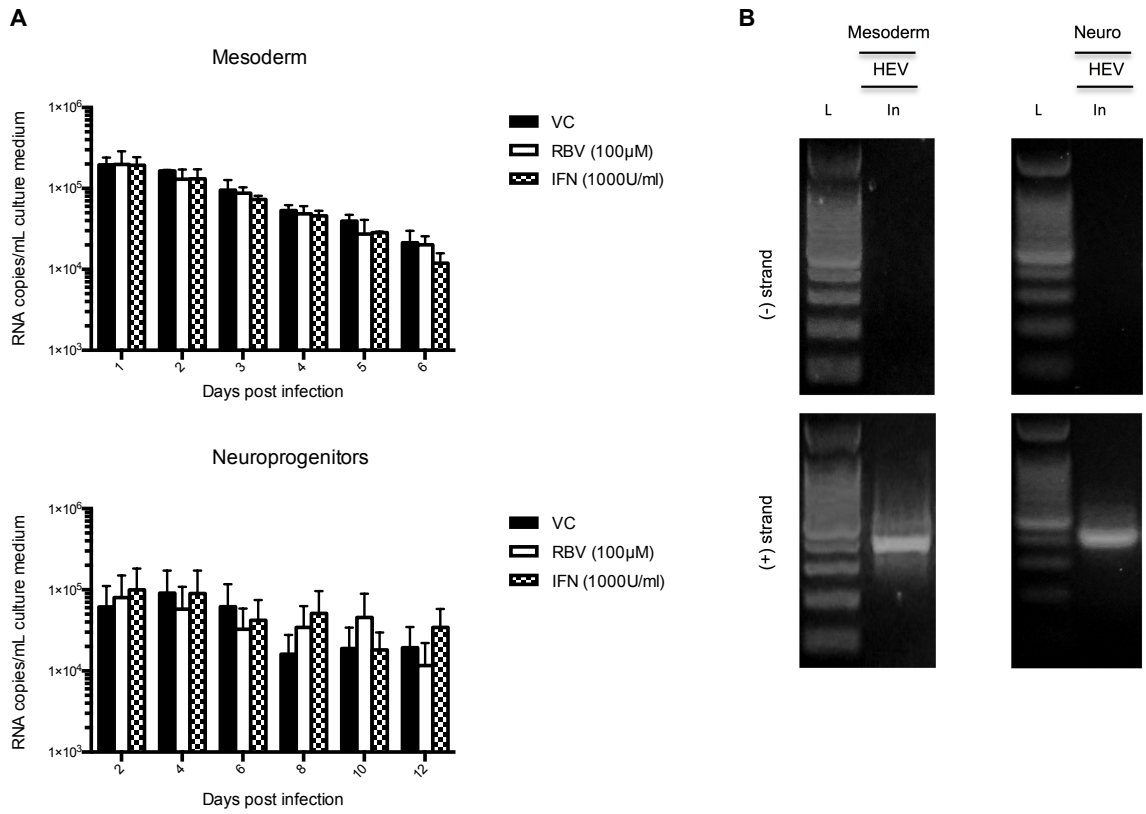

Figure 18. *Mesoderm and neuroprogenitors cells do not support HEV replication. (A) Neither ribavirin (RBV) nor interferon alpha (IFN) had an effect on the detected viral HEV viral RNA levels in hPSC-mesoderm and NPCs. Results represent the mean of three independent experiments ± SEM. (B) No negative strand RNA was detected in the lysates of hPSC-mesoderm and NPCs. Positive strand RNA was however detected in lysates of both cell types. Images are representative of three independent experiments. Ex, RNA extracted from culture medium samples; In, RNA extracted from lysates; L, 100 basepair DNA ladder (Promega).*

3.3.7 The entry of HEV is restricted to hPSC-derived hepatocytes

To examine whether the apparent failure to infect hESC-mesoderm and -NPCs with HEV occurs at the level of RNA replication or rather at an early step of the viral life cycle, both cell populations were transfected with the genotype 3 subgenomic p6/luc HEV replicon to examine whether these non-endodermal progeny cells support intracellular HEV replication. Interestingly, we demonstrated that both hESC-mesoderm and -NPCs do support replication of the HEV replicon, which for the hESC-NPCs, approached levels also observed in the Huh7 hepatoma control cell line (Figure 19). Furthermore, HEV

replicon replication was in all three cell lines efficiently inhibited by RBV treatment. This provides compelling evidence that the inability of HEV to enter into hESC-mesoderm and -NPCs is the main reason why these cells are not supporting the complete replication cycle of the HEV.

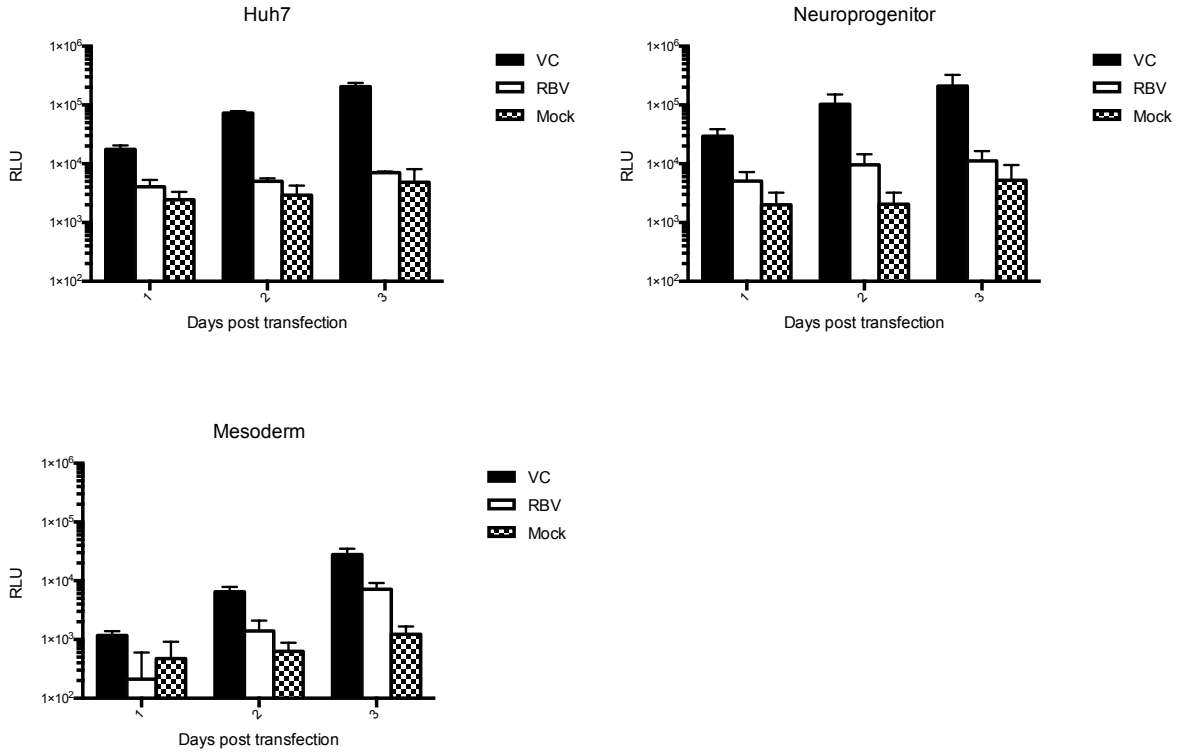

Figure 19. *Mesoderm and neuroprogenitors cells do support HEV replication upon transfection. Huh7 hepatoma, hPSC-mesoderm and NPCs were transfected with capped HEV p6/luc replicon RNA and Gaussia luciferase activity was measured in the culture medium at 1, 2 or 3 days post transfection in the presence or absence of ribavirin (RBV). Solid intracellular replication can be observed in all 3 cell types. Results represent the mean of three independent experiments ± SEM. VC, virus control; RBV, ribavirin; RLU, relative light units.*

3.4 Discussion

Hepatitis E is an emerging disease in developing and industrialized countries, that typically results in acute self-limiting hepatitis, but severe cases of both chronic and acute hepatitis have been reported (200). We here demonstrate that differentiated hepatocyte progeny from pluripotent stem cells support the complete replication of

HEV and thus forms a physiologically relevant model system to study the biology of HEV replication and strategies to inhibit viral replication. Moreover, the pluripotent nature of these stem cells, allowed us to generate mesodermal and ectodermal cell types. We demonstrate that these cannot be infected although they support intracellular replication of the virus. This cell cultures model(s) thus provide an elegant model to study the mechanism underlying the hepatotropism of HEV.

Interest in HEV research is growing due to the increasing medical importance of HEV infections, yet currently available cell culture models rely mostly on the use of hepatoma cells. These are however transformed cell lines that may not completely recapitulate the biology of HEV replication in the infected hepatocyte. Therefore, other cell sources are being explored for their ability to support HEV infection and replication.

In 2012, a porcine ESC line was differentiated into hepatocytes and successfully infected with swine fecal samples containing HEV of genotype 3 (177). Viral HEV RNA was detected until 35 days after infection in the supernatant and started to increase from day 8 onwards. Similarly to our findings, intracellular viral RNA levels remained constant in the swine ESC-derived hepatocytes, but were lower compared to what we observed in hPSC-hepatocytes. Negative strand RNA was also detected in porcine-derived hepatocytes, but because the low viral titer in the culture medium, reinfection experiments failed. By contrast, we detected in the supernatant of almost half of infected hESC-hepatocytes infectious viral progeny capable of re-infecting HepG2/C3A cells. Thus human PSC-hepatocytes support the entire HEV replication cycle and thus allow the study of the complete biology of HEV infection and replication in a physiologically relevant context.

The number of HEV RNA copies in the culture medium was lower in primary HEV infected hPSC-hepatocytes compared to HepG2/C3A cells that had been infected under the same conditions. Noteworthy, intracellularly and during the initial 4-6 days after infection RNA copy levels were comparable in both systems. We hypothesize that the difference in viral kinetics may in part be due to the fact that hepatoma cells continue to proliferate in culture, whereas hPSC-hepatocytes are chiefly quiescent. An alternative explanation might be that that the Kernow-C1 p6 strain, used in this study, was passaged 6 times in HepG2/C3A cells and may thus have been adapted to the HepG2/C3A cellular environment (112). The advantage of using pluripotent stem cells is that they can easily be obtained from different donors whereas hepatoma cell lines

are derived from a single donor. Availability of multiple donor hPSC-hepatocytes should allow further examination of specific virus-host interactions. Indeed, we found that there were differences in innate immune response following HEV infection of two PSC-derived hepatocyte progeny. Use of additional patient-specific iPSC lines should permit to further elaborate on this observation to fully understand virus-host interactions. Unfortunately, we were unable to cultivate HEV from a clinical plasma sample in our system, which confirms the challenging nature of HEV propagation in tissue culture.

In the hPSC-hepatocytes, HEV replication was efficiently inhibited by RBV and IFN, the only drugs currently available to treat HEV infections. Both drugs decreased (both intra- and extracellular) levels of viral RNA but did not fully eliminate the virus from the hPSC-hepatocytes. Complete eradication may require either longer treatment, more potent drugs, or may only be possible in the context of an intact immune response in the infected host.

Initially, infection studies were performed with genotype 3 HEV carrying an additional G1634R mutation. Comparison of the replication efficiency between wild type and mutant HEV in hESC-hepatocytes revealed the 1634R mutant might have some replication advantage in these cells as well, although the differences were not statistically significant. However, only supernatant collected from hESC-hepatocytes that had been infected with the mutant HEV was able to re-infect HepG2/C3A cells, which is consistent with the notion that 1634R mutant results in an increased viral fitness. hESC-hepatocytes might therefore also serve as a valuable tool to study the importance of mutations identified in HEV-infected patients that became non-responsive to ribavirin treatment.

In hPSC-mesoderm and NPCs, (-)ssRNA was not detectable and levels of (+)ssRNA decreased over time, suggesting that these cells do not support *in vitro* the full cycle of HEV infection and replication, which would be consistent with the tropism seen *in vivo*. However, as successful infection of a culture system depends not only on viral replication but also on the ability of the virus to enter the cell, i.e. infect the cell, we explored whether hPSC-mesoderm and NPCs support HEV subgenomic replication following transfection of the replicon in the host cells. hPSC-mesoderm and NPCs could support intracellular HEV replication, and therefore strongly suggest that entry is the limiting factor to allow the complete replication cycle of the HEV and may be the key determinant of HEV tissue tropism. Although some studies have demonstrated that HEV

infection can be associated with neurological symptoms such as Guillain-Barré syndrome (201-203), it is not clear whether such neurological symptoms are a direct or indirect consequence of HEV infection. In one study, HEV viral RNA was detected in cerebrospinal fluid of some patients and viral sequences found in the cerebrospinal fluid were different from those detected in serum (204). This may suggest that HEV variants with neurotropic capacity may indeed exist. The HEV infection model in PSC (from which we can generate many different mature cell types) will allow studying cell/tissue tropisms of HEV as well as the underlying mechanism.

In conclusion, pluripotent stem cells have the unique capacity to differentiate into any given cell type, which allows to study HEV infection not only in hPSC-hepatocytes but also to discover possible extrahepatic sites of HEV infection and replication. We here demonstrate that hPSC-mesoderm and NPCs support HEV replication, but only upon transfection of viral (replicon) RNA suggesting that the *in vivo* hepatotropism seen is likely due to the inability of HEV to enter non-hepatic cells. On the contrary, hepatocyte-like cells generated from hPSCs are a valuable alternative for hepatoma cell lines and human primary hepatocytes in the study of the viral biology of the HEV.

Chapter IV: The effect of DMSO on hepatocyte differentiation and subsequent infection with the hepatitis E and hepatitis B virus

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Planning to submit as:

Helsen N, Vanhove J, Tricot T, Boon R, Ordovas L, Abassi K, Urban S, Neyts J and Verfaillie C.M.

AUTHOR CONTRIBUTIONS: N.H. conceived the study, generated the data and wrote the paper. J.V. contributed to the DMSO experiments. T.T. and K.A. contributed to the HBV infection experiments. R.B. and L.O. made the RMCE line. S.U. provided the HepG2-NTCP cells and the HBV stock. J.N. and C.M.V. designed and supervised the study.

4.1 Introduction

Dimethyl sulfoxide (DMSO) is widely used as an amphiphilic solvent and a cryoprotectant. DMSO also functions as a free radical scavenger and influences the physiological properties of the lipid membrane. In addition, it was demonstrated that DMSO induces cell differentiation and retards dedifferentiation of cultured primary human hepatocytes (205, 206). DMSO is added in a concentration of 2% to the human hepatoma cell line, HepaRG, to induce differentiation and polarization (27). Therefore, DMSO has been included in varying concentrations ranging from 0.5 to 1% to numerous pluripotent stem cell (PSC) hepatocyte differentiation protocols (50, 186, 207). Chetty *et al.* previously demonstrated that the addition of DMSO to PSCs prior to differentiation increased the number of cells in early G1 phase and induced the differentiation of PSCs to all three germ layers, including endoderm (208). Another study confirmed that 0.5-0.6% DMSO downregulated the expression of pluripotency genes in human embryonic stem cells (hESCs) and improved endodermal differentiation (209). The mechanism of action whereby DMSO induces (endodermal) differentiation and prevents hepatocyte dedifferentiation remains however to be elucidated.

The hepatitis B virus has a remarkable host specificity and hepatotropism. HBV research was until recently hindered by the lack of a robust and efficient cell culture models. The HepaRG cell line was the first hepatoma cell line reported that supported the complete HBV replication cycle but only upon differentiation with 2% DMSO (142). In addition, it was shown that DMSO enhances the infection of primary human hepatocytes with HBV (210). Microarray analysis of undifferentiated HepaRG cells, DMSO-differentiated HepaRG cells and non-susceptible HuH7/ HepG2 cells identified NTCP as a candidate receptor for the HBV and overexpression of human NTCP in HuH7/HepG2 cells rendered these cells susceptible to HBV infection. Remarkably, 2.5% DMSO also increased the efficiency of HBV infection in NTCP overexpressing HuH7 and HepG2 cells. The exact role of DMSO during HBV infection remains unclear but it is thought that DMSO enhances the adsorption and cell association of HBV (210). DMSO induces the expression of NTCP and overall differentiation of HepaRG cells but might also enhance or maintain certain liver-enriched transcription factors that control HBV transcription (180).

Based on previous reports, we added 0.6% DMSO during the differentiation of PSCs towards endoderm and demonstrated an improvement in endodermal differentiation. However, DMSO is used at higher concentrations, ranging between 2 and 2.5%, in HepaRG and primary human hepatocytes cultures. Therefore, we hypothesized that addition of 2% might also enhance maturation of hepatoblasts from day 12 of differentiation onwards. We demonstrated that a more homogenous population of hepatocyte-like cells was generated using this protocol, yielding cells that were more susceptible to HEV infection. Moreover, the addition of DMSO significantly increased NTCP expression and preliminary studies suggest that this may render hepatocyte-like cells from PSCs susceptible to HBV infection. Finally, we performed a number of studies to determine how addition of DMSO enhances endoderm and hepatocyte-like cells differentiation from PSCs, but these studies were not conclusive.

4.2 Materials and methods

4.2.1 Cells and compounds. The fibroblast cell line BJ1 was previously reprogrammed to hiPSC by lentiviral transduction of *OCT4, c-MYC, KLF4* and *SOX2* (39). The hESC line H9 (WA09) was purchased from WiCell Research Institute (Madison, WI). Both hiPSC and hESC were maintained either on inactivated mouse embryonic fibroblasts (iMEF) in hESC medium (DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% KnockOut Serum Replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 1 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/mL bFGF (basic fibroblast growth factor) (Peprotech, Rocky Hill, NJ) or on human matrigel (BD) coated plates in Essential 8 medium (Life Technologies) in a humidified 5% CO_2 incubator at 37°C. HepG2-NTCP cells (a kind gift from Prof. Stephan Urban, University of Heidelberg) were cultured in DMEM (Gibco, Gaithersburg, MD) supplemented with 10% FBS, 1% penicillin-streptomycine (Gibco), 1% L-glutamine 200nM (Gibco) and 1% non-essential amino acids (Gibco)) in a humidified 5% CO_2 incubator at 37°C. Myrcludex B (MyrB), GFP-labeled MyrB and a mutated GFP-labeled MyrB were kindly provided by Prof. Stephan Urban. N-acetylcysteine was purchased from Sigma-Aldrich. Janus kinase (JAK) inhibitor was purchased from Millipore (Billerica, Massachusetts).

4.2.2 Optimization of the hepatocyte differentiation protocol by DMSO.

hESC and hiPSC were differentiated towards hepatocytes following a previously described protocol with minor modifications (169) (Figure 9). During differentiation DMSO (Sigma-Aldrich) was added in various concentrations ranging from 0.6% - 2% (Figure 22).

4.2.3 RNA extraction and quantitative reverse-transcription PCR (RT-qPCR).

For gene expression analysis, RNA was isolated from differentiated progeny cells by the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following manufacturer's procedures. Genomic DNA was removed using the On-Column DNase I Digestion kit (Sigma-Aldrich). The Superscript III First-Strand synthesis system (Invitrogen) was used for subsequent cDNA synthesis. qPCR was performed with the Platinum SYBR green qPCR supermix-UDG kit (Invitrogen) in a ViiA 7 Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene for normalization. Sequences of qRT-PCR primers are listed in (Table ***4***). Viral RNA was isolated from the supernatant using the NucleoSpin RNA virus kit (Macherey-Nagel, Düren) according to the manufacturer's instructions. Intracellular RNA from infected stem cell progeny was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). qRT-PCR for viral RNA was performed as previously described (194) (Table 2). Viral RNA detected in lysates was normalized to total RNA content as measured by spectroscopy (Nanodrop ND-1000, Thermo Fischer Scientific).

Gene	Forward	Reverse
GAPDH	TCAAGAAGGTGGTGAAGCAGG	ACCAGGAAATGAGCTTGACAAA
AFP	TGAGCACTGTTGCAGAGGAG	GTGGTCAGTTTGCAGCATTC
ALB	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA
AAT	AGGGCCTGAAGCTAGTGGAT	TCCTCGGTGTCCTTGACTTC
HNF4α	ACTACGGTGCCTCGAGCTGT	GGCACTGGTTCCTCTTGTCT
NTCP	ATCGTCCTCAAATCCAAACG	CCACATTGATGGCAGAGAGA
G6PD endo	ACAGCGTCATGGCAGAGC	TCTTCTTCTTGGCCAGGTCAC
G6PD exo	GTGACCTGGCCAAGAAGAAG	AGGTGGAGGGCATTCATGT
OCT4	TCGAGAAGGATGTGGTCCGA	GCCTCAAAATCCTCTCGTTG
SOX2	TGGCGAACCATCTCTGTGGT	CCAACGGTGTCAACCTGCAT
NANOG	CCTGTGATTTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT
CXCR4	CACCGCATCTGGAGAACCA	GCCCATTTCCTCGGTGTAGTT
FOXA2	AGGAGGAAAACGGGAAAGAA	GGTGCTTGAAGAAGCAGGAG
EOMES	AACAACACCCAGATGATAGTC	TCATAGTTGTCTCTGAAGCCT
MIXL1	GGATCCAGGTATGGTTCCAG	CATGAGTCCAGCTTTGAACC

Table 4. *RT-qPCR primer list.*

4.2.4 Flow cytometry. On day 4, cells were detached with trypsin 0.05% and stained with 1 μg/mL anti-CXCR4-PE (BD Biosciences, San Jose, CA) and 2 μg/mL anti-cKIT-APC antibody (BD Biosciences) for 15 minutes at room temperature. Afterwards cells were washed and analyzed by flow cytometry analysis using a FACS-Canto (BD). For intracellular AAT flow cytometry staining, a single cell suspension was made by liberase treatment (Roche, Basel, Switzerland) followed by fixation with 4% PFA for 15 min. at RT. Next, cells were permeabilized with 0.1% saponin and blocked with 10% goat serum (Dako, Glostrup, Denmark) for 45 min. at RT. Afterwards cells were stained with 0.0625μg/200μL/10^6 cells anti-AAT antibody (Dako) or a rabbit IgG isotype control (BD Pharmingen) for 1h at RT. After 1h, a secondary Alexa Fluor 647 antibody (1:1500) (Invitrogen) was used for 30 min. at RT. Cells were analyzed by flow cytometry analysis using a FACS-Canto (BD).

4.2.5 Immunofluorescence. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min. at room temperature (RT), permeabilized with 0.2% Triton X-100, blocked with 5% normal donkey serum (Jackson Laboratory, Bar Harbor, ME) and stained overnight at 4°C with primary antibodies and isotype controls Table 5). Afterwards, cells were incubated with appropriate secondary antibodies together with Hoechst (Sigma-Aldrich) for 60 minutes at RT). Images were taken by the AxioimagerZ.1 fluorescence microscope.

Antibody	Catalog number	Company	Dilution
SOX17	AF1924	R&D	1:200
HNF4α	Ab41898	Abcam	1:200
NTCP	HPA042727	Sigma	1:500

Table 5. *Antibody list used for immunofluorescence assays.*

4.2.6 Recombinant mediated cassette exchange (RMCE). A master cell line containing an FRT-flanked CAGGS-GFP-hygromycin/Thymidine Kinase (TK) cassette introduced in the *AAVS1* locus, using homologous recombination, was previously generated in the lab by Dr. Ordovas (211). Two million cells were nucleofected with 6μg of the donor plasmid containing an FRT-flanked inducible G6PD-puromycine cassette together with 3μg of the Flippase expression vector using the Nucleofector solution kit 2 (Amaxa)(Figure 24). Nucleofected cells were plated on puromycin resistant iMEFs and 2 days after nucleofection puromycin selection was started with increasing concentration of puromycin ranging from 100-200ng/mL for 6 days. As negative selection, four days after the start of puromycin selection 0.5 μM 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodouracil (FIAU) was used for 7 days. After selection, puromycin/FIAU resistant colonies were picked, expanded and characterized.

4.2.7 Albumin and AAT ELISA. Enzyme-linked immunosorbent assay (ELISA) for albumin and AAT was performed according to the manufacturer's procedure (Bethyl, Montgomery, TX). Briefly, on day 20 of the hepatocyte differentiation protocol, supernatants were collected and incubated with a primary albumin or AAT antibody for 60 min. at RT. Afterwards an HRP-detection antibody was added for 60 min. followed by TMB-peroxidase solution for 15 min. at RT. The reaction was stopped by a 0.18M H_2SO_4 solution and the OD was measured on a microplate reader at a wavelength of 450 nm. A standard curve was used to quantify the amount of albumin and AAT secretion.

4.2.8 Cell cycle analysis. Day 20 PSC-progeny were harvested with liberase (Roche, Basel, Switzerland), washed with 1X PBS, suspended in 1X PBS + 0.1% glucose and immediately fixed with ice cold 70% ethanol for 1 hour. After fixation, cells were spun down, washed with 1X PBS and resuspended in the staining solution containing

propidium iodide and pyronin Y for 30-45 minutes at 37°C, and cells subsequently analyzed by flow cytometry analysis using a FACS-Canto (BD).

4.2.9 HEV viral inoculation. Day 20 PSC-derived hepatocyte progeny was infected with 300 µL HEV stock diluted to 3 x 10^7 viral RNA copies/mL per well and incubated for 24h at 37°C in a 5% CO2 humidified incubator. After 24h, the inoculum was removed and cells were washed 5 times with 500 µL of DMEM, before addition of 500 µL of hepatocyte differentiation medium. Medium was changed every other day by collecting 300 µL and replacing it with 350 µL fresh hepatocyte differentiation medium

4.2.10 Myrcludex B staining. Stem cell-derived hepatocytes and HepG2-NTCP cells were cultured on cover slips and at incubated with 400nM GFP-labeled Myrcludex B for 30 minutes at 37°C. After incubation, cells were washed 3x with PBS/2% bovine serum albumin and fixed with 4% PFA for 15 minutes at room temperature. Afterwards cells were washed 3x with PBS, stained with Hoechst (Sigma-Aldrich) and mounted. Images were taken by an AxioimagerZ.1 fluorescence microscope.

4.2.11 HBV viral inoculation. HepG2-NTCP cells (kindly provided by Professor S. Urban) were seeded, one day prior to infection, at a density 20.000 cells per well of a 96 well plate. Both HepG2-NTCP and day 16 stem cell-derived hepatocytes were infected with a 1:100 (≈ 8 x 10^9 genome equivalents) and 1:10 dilution (≈ 8 x 10^{10} genome equivalents) of the HBV stock (produced by the laboratory of Professor S. Urban), respectively. The viral inoculum was supplemented with 2.5% DMSO and 8% polyethylene glycol (PEG). Cells were incubated for 24 hours at 37°C. After viral inoculation, medium was replaced by HepG2 medium or liver differentiation medium (169). Medium was changed every other day until 6 days post infection. Cells were treated from the inoculation until the end of the experiment with 200nM of Myrcludex B (kindly provided by Professor S. Urban) or with 1µM Janus Kinase (JAK) inhibitor (Millipore).

4.2.12 HBcAg staining. On day 6 post infection, cells were fixed with 4% PFA for 15 minutes at room temperature. Afterwards, cells were blocked and permeabilized

overnight at 4°C with 3% Bovine Serum Albumin, 2% Fetal Calf Serum and 0.2% Tween-20. The next day, cells were stained for 90 minutes at room temperature with a hepatitis B core antigen (HBcAg) antibody (Dako) in a dilution of 1:750. After incubation with the primary antibody, cells were washed 3X with PBS and stained for 1 hour at room temperature with a complementary secondary antibody and Hoechst. Images were taken either using an InCellAnalyzer (GE Healthcare Life Sciences, Diegem, Belgium).

4.3 Results

4.3.1 DMSO improves definitive endoderm formation from hPSCs

DMSO was added at a concentration of 0.6% to the differentiation protocol from day 0 onwards (Figure 20A). The effect of DMSO on definitive endoderm formation was evaluated by RT-qPCR, flow cytometry and immunofluorescence staining. Addition of DMSO significantly enhanced downregulation of pluripotency genes such as *OCT4*, and significantly increased a number of endodermal markers transcripts in both hESC and hiPSC-derived endodermal cells (Figure 20B). In addition, the frequency of CXCR4/cKIT double positive cells increased to approximately 70-80% (Figure 21A). The improvement of definitive endoderm by DMSO was further confirmed by SOX17 immunofluorescence staining (Figure 21B). The effect of DMSO was more pronounced when evaluating endodermal protein expression compared with gene transcript levels.

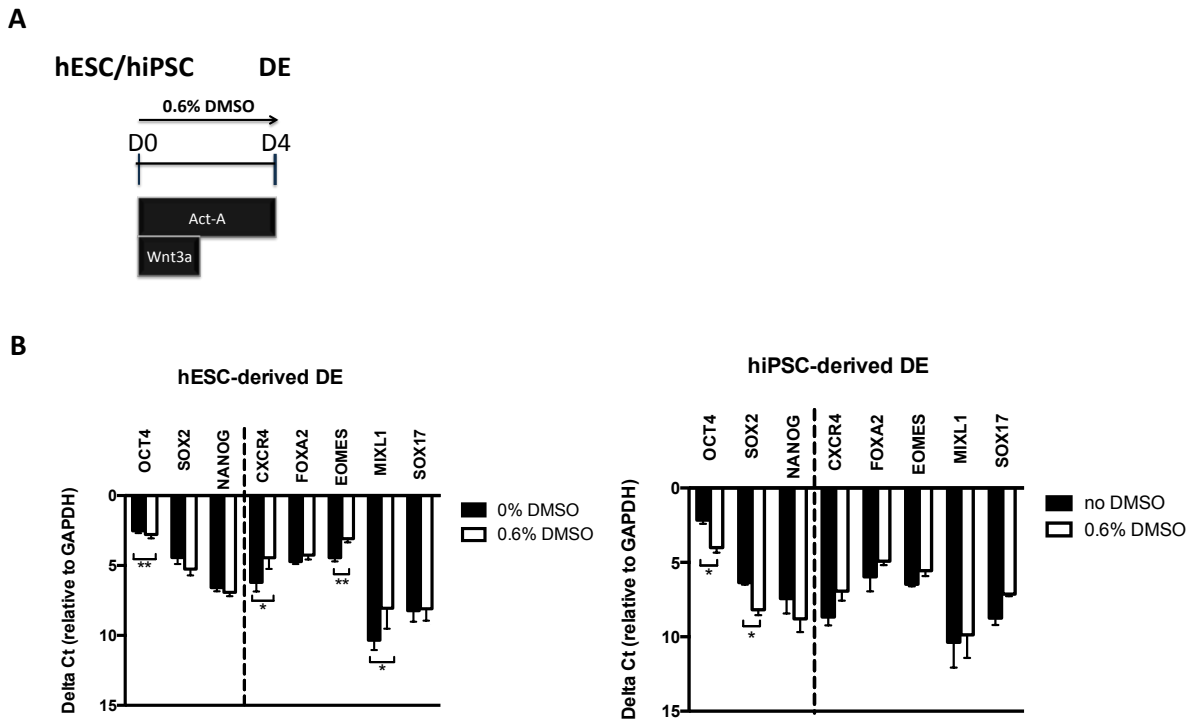


Figure 20. *DMSO improved endoderm formation. Differentiation of hPSCs to definitive endoderm was performed with or without addition of 0.6% DMSO (A) Differentiation protocol. (B) RT-qPCR analysis of pluripotency genes and definitive endoderm genes (n=3; *: p=0.05%)).*

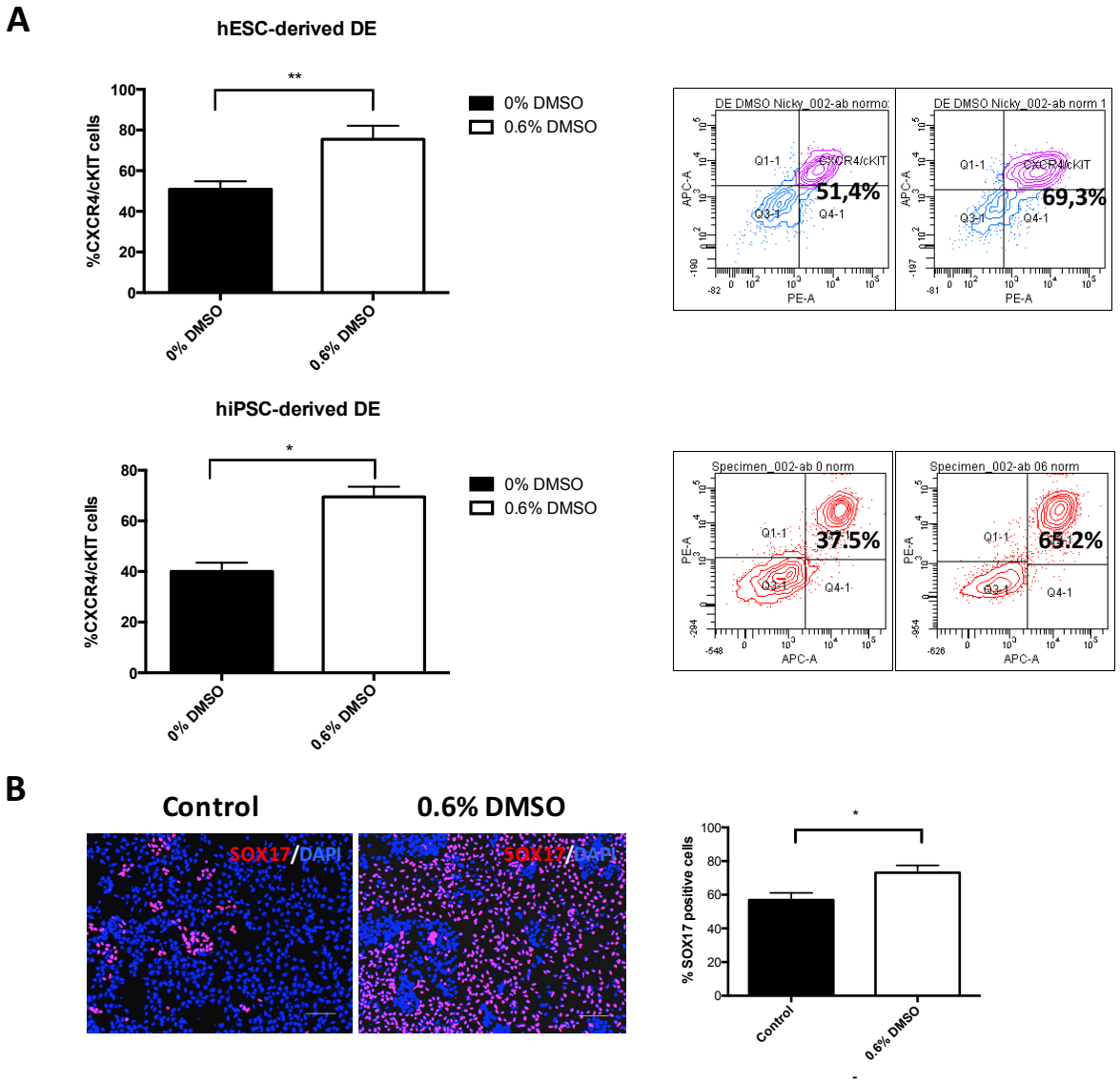
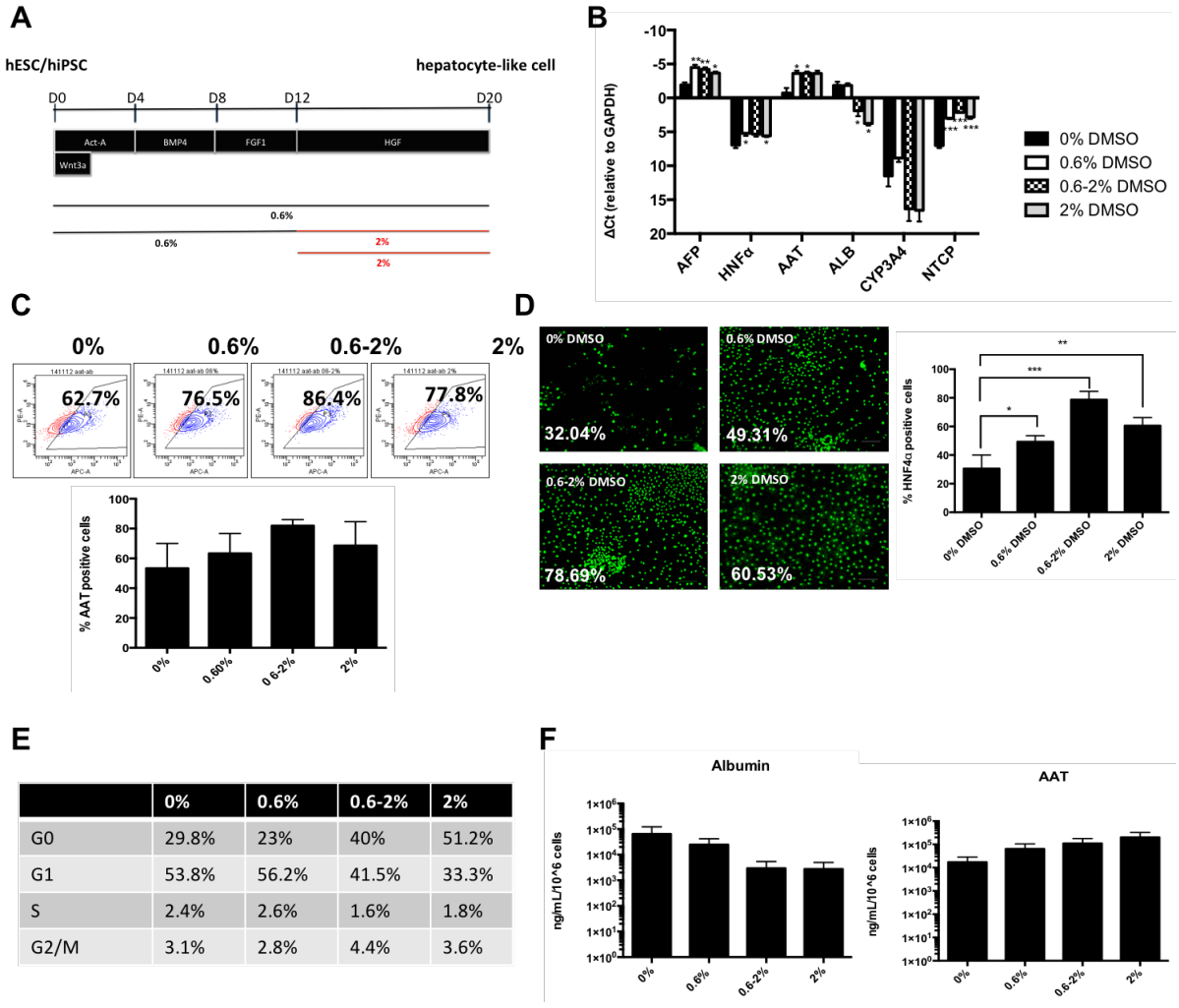


Figure 21. *DMSO improved the generation of definitive endoderm cells. Differentiation of hPSCs to definitive endoderm was performed with or without addition of 0.6% DMSO. (A) FACS analysis with antibodies against CXR4 and cKIT on day 4 hPSC progeny (n=3; *p=0.5%). (B) Immunofluorescence staining with antibodies against SOX17: left panel representative example; right panel: quantification of SOX17 positive cells (n=3; *p=0.5%).*

4.3.2 Effect of DMSO on the differentiation of hepatocytes from hPSCs

We next examined the effect of DMSO on hepatocyte differentiation by day 20. The first protocol was the standard protocol without DMSO (0% DMSO), published by our group (144, 169); the second protocol contained 0.6% DMSO from day 0 until the end of the differentiation (0.6% DMSO); while in the third protocol 0.6% DMSO was added from

day 0 until day 12 and then further increased to a concentration of 2% until day 20. In the last and fourth protocol, 2% DMSO was only administrated only from day 12 until the end of differentiation (2% DMSO) (Figure 22A). The rationale for increasing in DMSO concentration to data 2% from day 12 (hepatoblasts stage) was based on the published studies demonstrating that maturation of HepaRG cells is accomplished by addition of 2% DMSO, and the ability of 2% DMSO to prevent too some extent the dedifferentiation of primary human hepatocytes (27, 206).



	0%	0.6%	0.6-2%	2%
G0	29.8%	23%	40%	51.2%
G1	53.8%	56.2%	41.5%	33.3%
S	2.4%	2.6%	1.6%	1.8%
G2/M	3.1%	2.8%	4.4%	3.6%

Figure 22. *Late-term effect of DMSO on PSC-hepatocyte differentiation. (A) Various concentrations of DMSO were added during the hepatocyte differentiation protocol. (B) RT-qPCR analysis for AFP, HNF4α, AAT, ALB, CYP3A4 and NTCP (n=3; *:p=0.05%). (C) Flow cytometry analysis for AAT (representative example of n=3; mean ± SEM of the three studies). (D) Immunofluoresence staining for HNF4α (representative example of n=3; quantification of the three studies, *:p=0.05%). (E) Cell cylce analysis on day 20 using propidium iodide and pyronin Y to define the frequency of cells in G0, G1, S and G2/M of*

the cell cycle (n=2). (F) ELISA on supernatant of day 20 hPSC progeny for albumin and AAT (n=3).

RT-qPCR analysis for hepatocyte markers demonstrated that *AFP, AAT, HNF4α* and *NTCP* were all upregulated by the addition of DMSO. The gene expression of albumin remained similar when 0.6% DMSO was added to the differentiation medium compared to cultures without DMSO. However, *ALB* transcripts were significantly decreased when the concentration was increased to 2% DMSO. Expression of *CYP3A4* was the highest in the 0.6% DMSO protocol but decreased as well with when 2% DMSO was used, even though these differences were not statistically significant. (Figure 22B). In line with the RT-qPCR studies, ELISA demonstrated, although not significant, a decrease in albumin secretion when 2% DMSO protocols were used, while AAT secretion was increased in all DMSO protocols (Figure 22F). In addition, immunostaining demonstrated a significant increase in the frequency of HNF4α positive cells in the DMSO protocols rendering a more homogenous population of hepatocytes (Figure 22D). The improvement of homogeneity of the differentiation was further demonstrated by AAT flow cytometry. In accordance with the gene expression and ELISA data, the frequency of AAT + cells increased, altough not signifciant, by the addition of DMSO with the highest of frequency of AAT + cells (approximately 85%) in the 0.6-2% DMSO protocol (Figure 22C). Cell cycle analysis of day 20 hPSC progeny, demonstrated that more cells treated with 2% DMSO were in G0 phase of the cell cycle compared with cultures treated with 0% or 0.6% DMSO.

3.3.6 N-acetylcysteine and induction of glutathione have a similar effect on definitive endoderm formation as DMSO

DMSO can act both as an oxidant of free thiol groups in proteins or as a reducing agent, scavenging free hydroxyl and peroxy radicals. The overall effect of DMSO is however highly cell type and concentration dependent (212)(213). Because of the free radical scavenger and antioxidant properties, we tested if another well-established antioxidant, N-acetylcysteine (NAC), would phenocopy the effect of DMSO on PSC-hepatocyte differentiation (205). NAC is a precursor of L-cysteine and glutathione, and scavenges free radicals (214, 215). NAC was added to the medium from day 0 to 4 at a

concentration of 2.5mM (Figure 23A). The addition of NAC significantly improved the generation of definitive endoderm to a similar extent as DMSO. The frequency of both CXCR4/cKIT double positive cells and SOX17 positive cells was significantly increased as well the expression of transcripts for the endodermal markers such as *CXCR4, EOMES* and *MIXL1*, while pluripotency markers were significantly more downregulated (Figure 23). However, improved generation of definitive endoderm by NAC did not lead to an improved differentiation of day 20 hepatocyte-like cells. Neither the administration of NAC from day 0 until day 4 or from day 0 until day 20 influenced the later time-points of the hepatocyte differentiation as was demonstrated by RT-qPCR (Figure 23E).

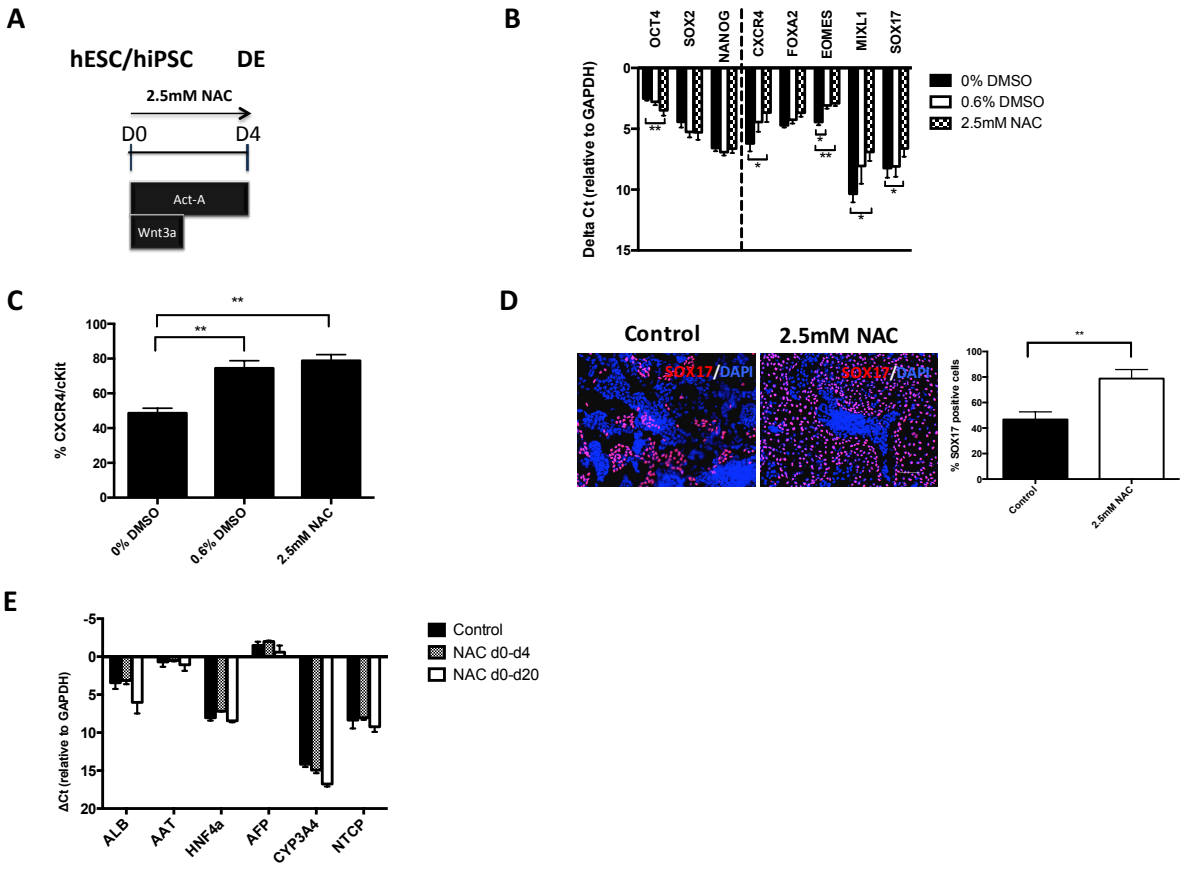


Figure 23. *Effect of NAC on definitive endoderm formation. (A) 2.5mM NAC was added to differentiation from day 0 until day 4. (B) RT-qPCR for pluripotency genes and definitive endoderm markers (n:6; *: p=0.05%). (C) FACS analysis for CXCR4 and CKIT double positive cells in cultures supplemented with or without 0.6% DMSO or 2.5mM NAC (n=3; *: p=0.05%). (D) Immunostaining for SOX17 and quantification (n=3; left panels representative image; right panel quantification; *: p=0.05%).*

Given rationale first, we examined the effect of glucose-6-phosphatase dehydrogenase (G6PD) overexpression. We created a doxycycline inducible G6PD overexpression cell line by recombinant mediated cassette exchange (RMCE) in the H9 hESC line engineered with an FRT flanked cassette in the AAVS1 locus (211). Overexpression of G6PD increased reduced glutathione (GSH) content in the cell. Reduced glutathione is the main antioxidant produced in cells and neutralizes reactive oxygen species and free radicals (Figure 24). However, additional experiments are required to measure the balance between reduced and oxidized glutathione content in the cell.

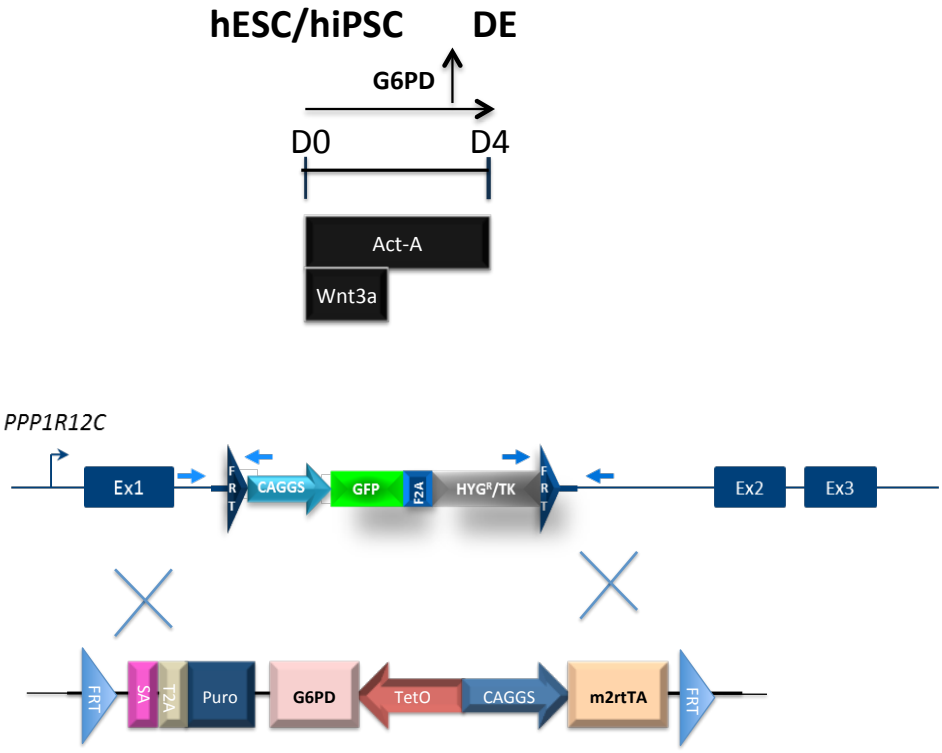


Figure 24. *Overexpression of G6PD. A G6PD doxycycline inducible cell line was created by using flippase recombinant mediated cassette exchange in the AAVS1 locus of H9 hESC.*

Overexpression of G6PD from day 0 until day 4 was confirmed by RT-qPCR and western blot (Figure 25 A&B). Although not significantly, upon doxycycline treatment the frequency of CXCR4/cKIT double positive cells was increased as was the frequency of SOX17 positive cells (Figure 25 C&E). Additionally, transcripts for definitive endoderm makers were significantly higher in response to G6PD overexpression (Figure 25D). The effect of overexpression of G6PD on day 20 hepatic progeny has not yet been assessed.

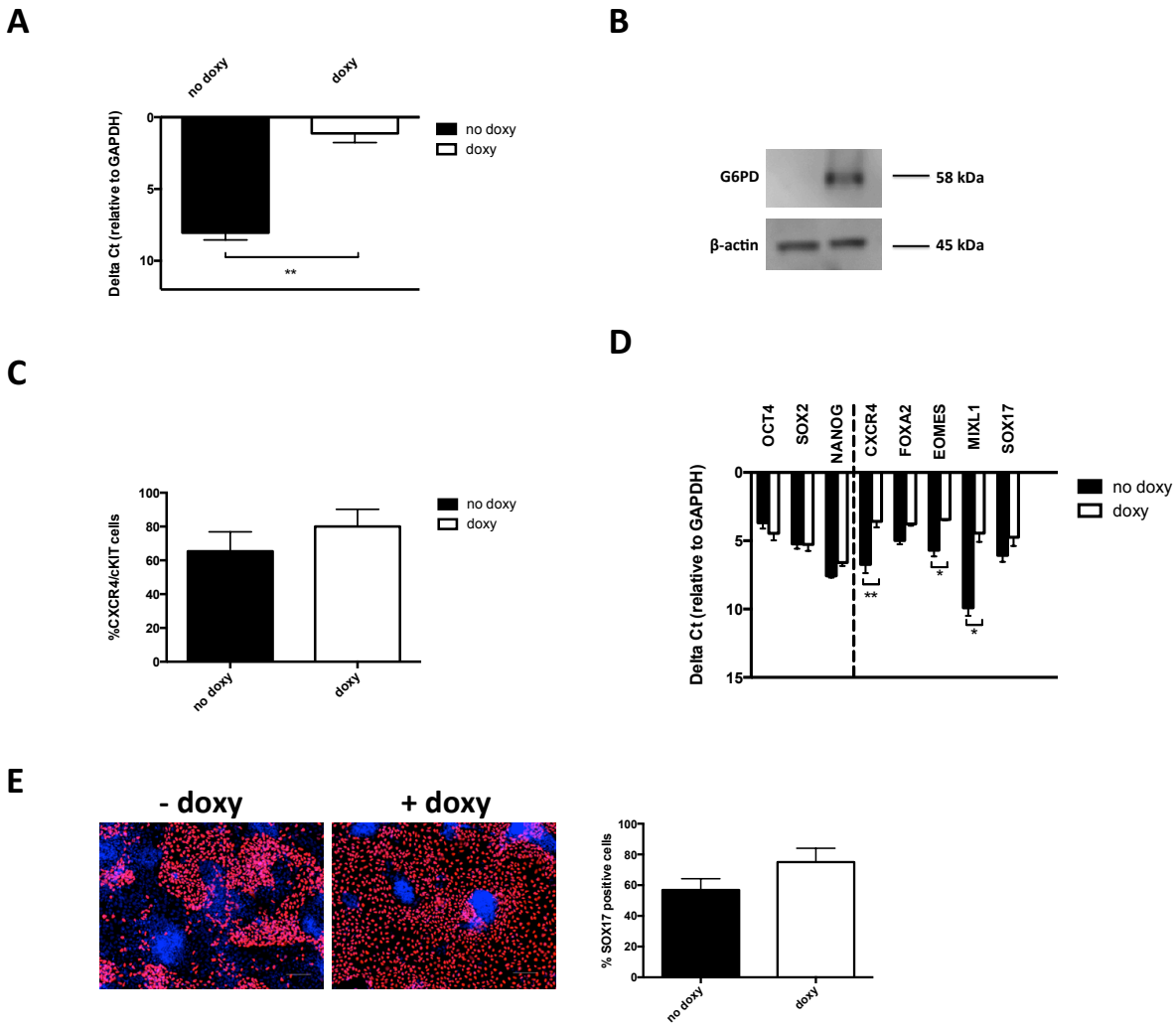


Figure 25. *Effect of G6PD overexpression on definitive endoderm formation. (A&B) RT-qPCR and western blot for G6PD in cells treated with or without doxycycline. (C) FACS analysis for CXCR4/cKIT double positive cells (n=3; *: p=0.05%). (D) RT-qPCR analysis for definitive endoderm genes on day 4 of ESC progeny treated with or without doxycycline (DOXY) during endoderm differentiation (n=3; *: p=0.05%). (E) Immunostaining for SOX17 and quantification (n=3, representative image).*

4.3.4 Infection of DMSO-treated hepatocytes with the hepatitis E virus

As addition of DMSO improves the homogeneity of hPSC hepatocyte-like cells (Figure 22), we re-examined the susceptibility of hPSC progeny differentiated without DMSO or with 0.6% DMSO from day 0 until day 12 following the addition of 2% DMSO from day 12 until day 20 (Figure 22) to HEV infection. Intracellular and secreted HEV RNA levels, quantified by RT-qPCR were significantly increased over the course of 12 days following infection in cells treated with DMSO (Figure 26). The improved homogeneity of hPSC-hepatocyte-like cell progeny in the presence of DMSO is therefore beneficial for the HEV infection efficiency of hPSC hepatocyte-like cells.

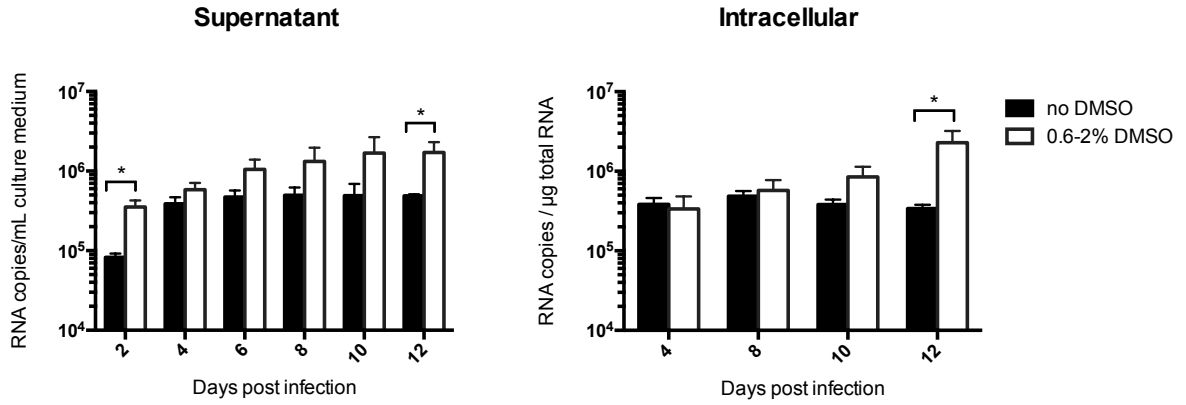


Figure 26. *DMSO enhances the susceptibility of stem cell-derived hepatocytes with the HEV. RT-qPCR for viral RNA levels in the supernatant and in the cell lysates over a period of 12 days (n=3; *: p=0.05%).*

4.3.5 Effect of DMSO on NTCP expression

NTCP was recently identified as the key receptor for HBV entry and its expression is required for successful HBV infection in hepatoma cells or primary human hepatocytes *in vitro* (126, 180). We demonstrated that the addition of DMSO significantly upregulated transcripts for *NTCP*, which was most pronounced in the 0.6-2% DMSO protocol (Figure 22B). Immunofluorescence staining also demonstrated that approximately 70.1% (±5.47) of the cells were NTCP positive following treatment of hPSC hepatocyte-like cell progeny using 0.6-2% DMSO protocol, whereas only 4.7% (±3.81) hepatocyte-like cells from cultures without DMSO stained positive for NTCP.

NTCP was as well detected by western blot, both in hESC and hiPSC – derived hepatocytes on day 16 of the differentiation in the presence of 0.6-2% DMSO (Figure 27A&B), even if levels remained significantly lower than those in NTCP overexpressing HepG2 cells (HepG2-NTCP). This is however not surprising as HepG2-NTCP cells were generated by lentiviral vector–mediated overexpression of NTCP. Time-course analysis of PSCs differentiated in the presence of 0.6-2% demonstrated that NTCP is significantly upregulated from day 8 onwards with the highest expression of NTCP on day 16 of the differentiation (Figure 27C).

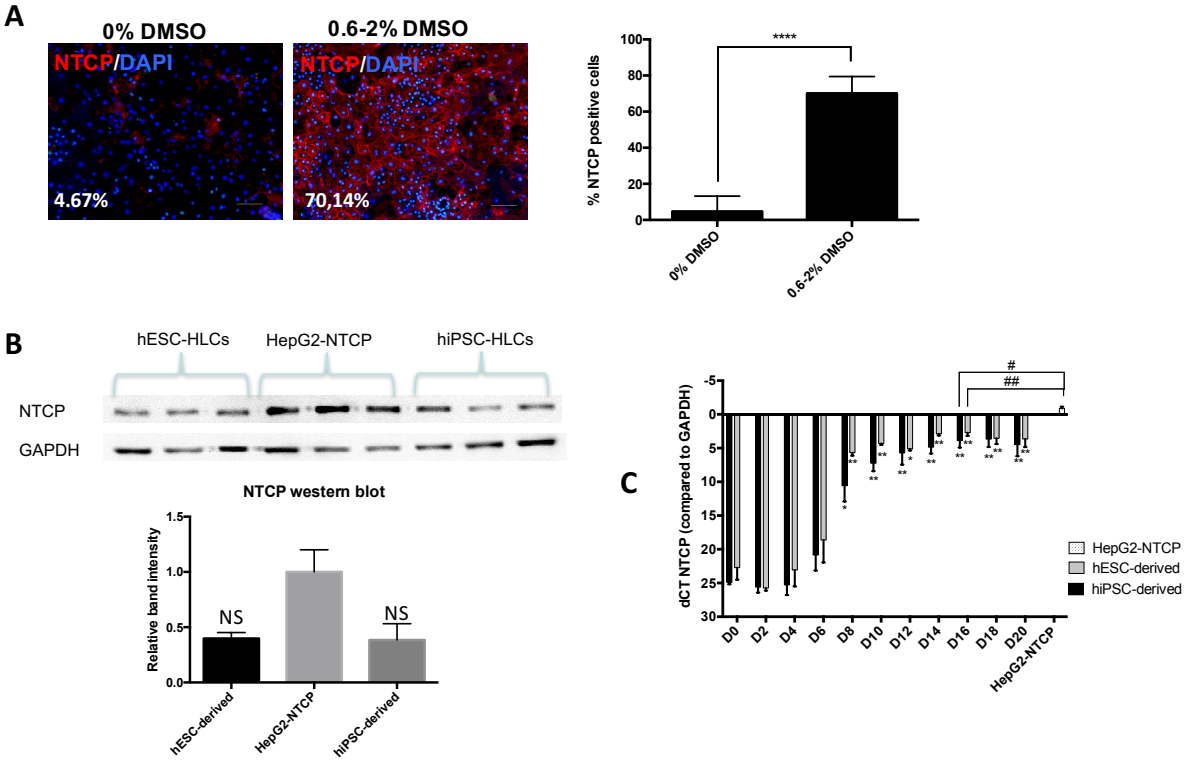


Figure 27. *Effect of DMSO on NTCP expression. (A) Immunofluorescence staining for NTCP of day 20 cells treated with or without DMSO treatment (n=3; *: p=0.05%). (B) Western blot analysis for NTCP protein in hESC and hiPSC derived progeny on day 16. HepG2 cells (HepG2-NTCP) were used as a positive control (n=3, lower graph shows quantification; NS= non-significant: p>0.05%). (C) RT-qPCR time-course analysis for NTCP transcripts in hESC and hiPSC progeny in comparison with HepG2-NTCP cells (n=3; * relative to day 0 (D0): p=0.05%; # relative to HepG2-NTCP: p=0.05%).*

4.3.6 Infection of stem cell-derived hepatocytes with the hepatitis B virus

Initially, we examined the binding of GFP-labeled Myrcludex B (MyrB-GFP) to HepG2-NTCP cells and day 16 PSC-hepatocyte-like cells. Myrcludex B is a peptide that corresponds to the pre-S1 domain of the large envelop protein of HBV which was demonstrated to reversibly bind to the NTCP receptor and to block entry of HBV (126, 180). As a control, cells were incubated with a GFP-labeled mutated form of MyrB (MyrB-mut-GFP) that is not capable of binding to the NTCP receptor. As demonstrated by immunofluorescence staining, NTCP was abundantly expressed by both, HepG2-NTCP cells and 0.6-2% DMSO treated day 16 hPSC-HLCs. MyrB stained both HepG2-NTCP cells and hPSC-HLCs. However, staining of hPSC-HLCs with MyrB was lower compared to HepG2-NTCP cells (Figure 28). MyrB + cells were not quantified due to technical issues.

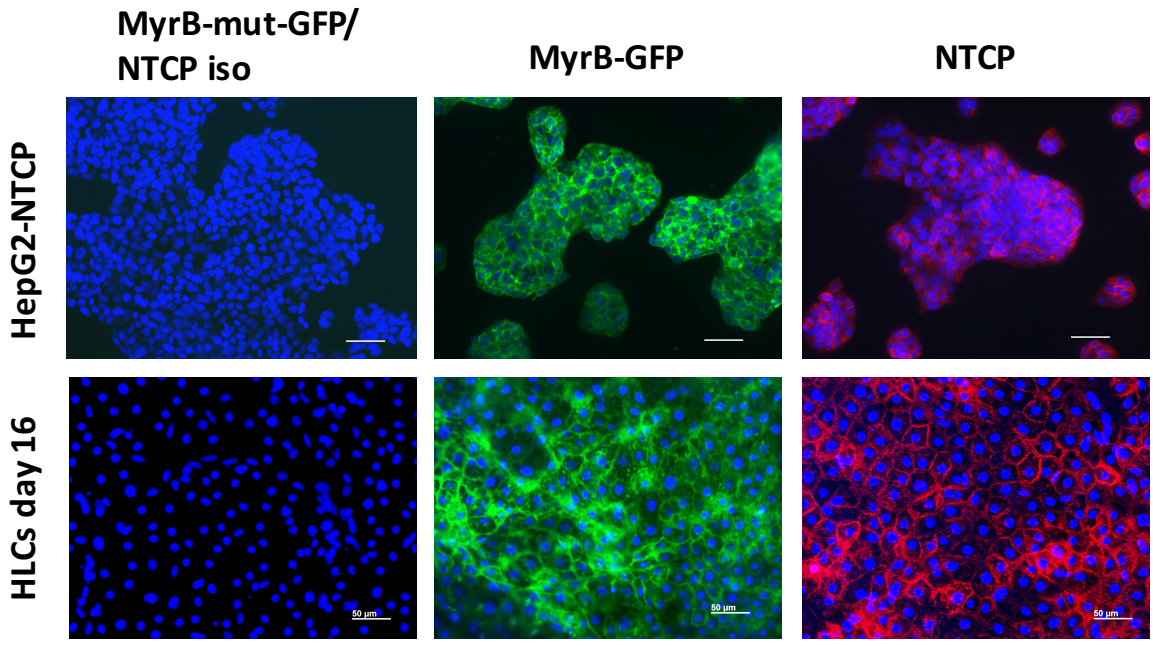


Figure 28. *Immunofluorescence with an antibody against NTCP and GFP-labeled MyrB of HepG2-NTCP cells and day 16 hPSC-derived HLCs (n=3; representative example shown).*

As MyrB was shown to bind DMSO differentiated hPSC-derived HLCs, we investigated their susceptibility to HBV infection. Overexpression of NTCP in the HepG2-NTCP cells was previously shown to allow highly efficient HBV infection, and these cells were

therefore used a positive control (180). Both HepG2-NTCP cells and hPSC-derived HLCs (differentiated with the 0.6-2% DMSO protocol) were inoculated with a concentrated HBV stock for 24 hours in the presence of 2.5% DMSO and 8% PEG. Presence of the hepatitis B core antigen (HBcAg) was assessed 6 days after infection by immunofluorescence staining. HepG2-NTCP cells and PSC-derived HLCs were susceptible to HBV infection. In addition, infection of PSC-derived HLCs with HBV was reduced by treatment with 200nM of the entry blocker MyrB (Figure 29).

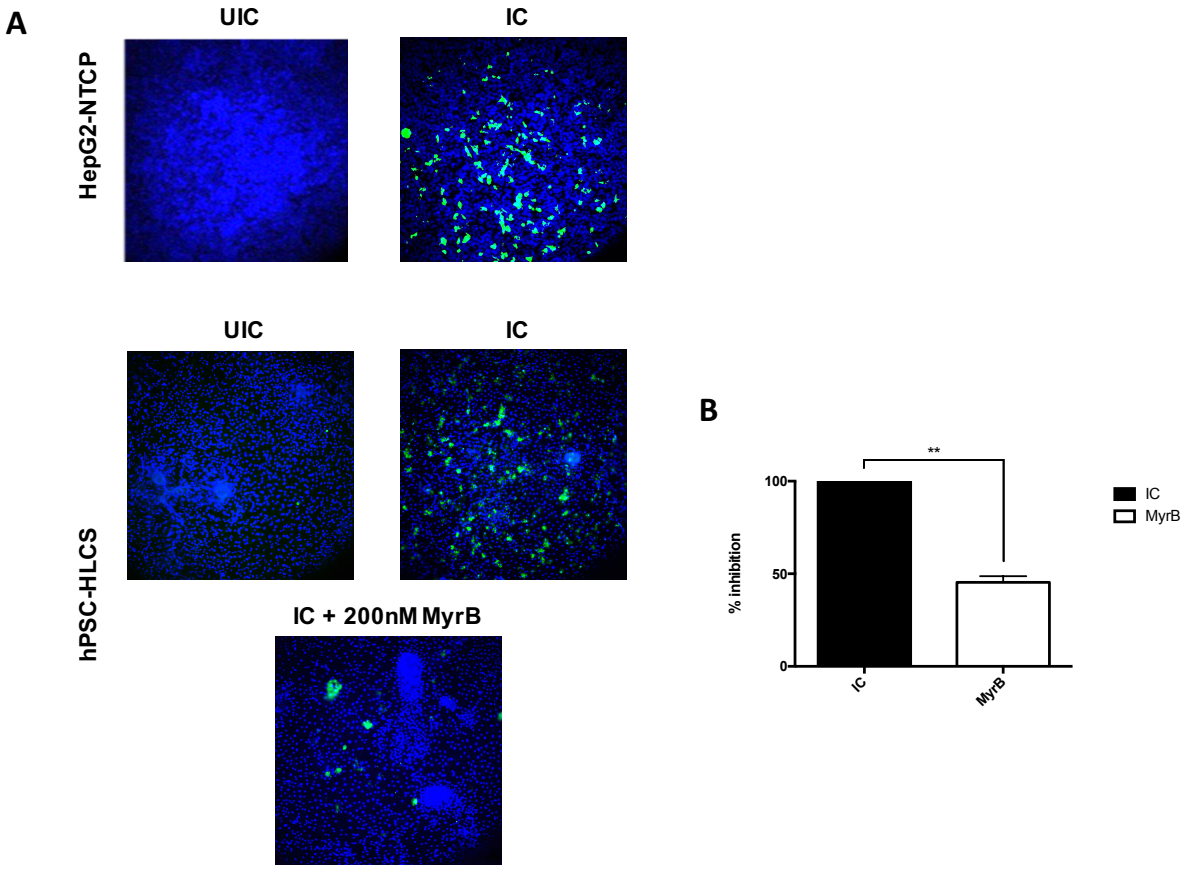


Figure 29. *Infection of HepG2-NTCP and hPSC-derived HLCs with HBV. (A) Immunostaining demonstrated the presence of HBcAg in both infected (IC) HepG2-NTCP and hPSC-HLCs. HBV infection of hPSC-HLCs was inhibited by treatment with 200nM MyrB. Uninfected cells (UIC) were used as negative control (n=3) (representative image). (B) Percentage of HBV inhibition by MyrB treatment was quantified by the InCell Analyzer Software (n=3; *: p=0.05%).*

4.4 Discussion

The role of dimethyl sulfoxide (DMSO) in cell differentiation was established many years ago. In 1975, DMSO was shown to induce the differentiation of erythroleukemia cells, and later on also to further induce the differentiation of neuroblastoma cells among others (216, 217). In addition, Isom *et al*., demonstrated for the first time that the addition of 2% DMSO could delay the dedifferentiation of primary rat hepatocytes *in vitro* (218). More recently, DMSO was found to be required to differentiate the bipotential hepatocarcinoma cell line, HepaRG, and several studies were published demonstrating a beneficial effect of DMSO on the differentiation of pluripotent stem cells toward hepatocyte-like cells (27, 50, 168, 186, 207, 209, 219).

We here demonstrated that the addition of 0.6% DMSO during endoderm differentiation generated a more homogenous population of definitive endoderm cells. DMSO is involved in different cellular processes and until now, the mechanism of action whereby DMSO affects differentiation of hepatocytes from hPSCs has not been unraveled. One possible mechanism might be that DMSO affects the permeability of the membrane lipid bilayer, which might enable transport of media components into the cell. In line with this hypothesis is the notion that DMSO might function as a carrier of nutrients and supplements (205). Another property of DMSO is its scavenger function of free radicals (205). We replaced DMSO by N-acetylcysteine (NAC), a well-studied antioxidant, and examined whether possible scavenging of DMSO was involved in the improvement of definitive endoderm differentiation. Addition of NAC indeed improved the formation of definitive endoderm cells to the same extent as DMSO. Moreover, the overexpression of G6PD, the rate-limiting enzyme for glutathione synthesis, improved as well the differentiation of hPSCs towards endoderm. We could however not detect a difference in reactive oxygen species (ROS) in non-treated versus DMSO or NAC treated cells (data not shown). Additional experiments will be required to further elucidate the involvement of the antioxidant response PSC-endoderm differentiation.

As DMSO is added at a concentration of 2% to prevent de-differentiation of primary hepatocyte cultures and to induce hepatocyte differentiation from HepaRG cells, we also examined the effect of 0.6 and 2% DMSO during the hepatocyte maturation from hepatoblasts between day 12 and 20 of the differentiation assay. When either 0.6 or 2% DMSO was present, the number of AAT and HNF4α positive cells was

significantly increased, with in addition an increase in AAT secretion. In contrast, upon addition of 2% DMSO, the expression of *CYP3A4* and *albumin* were downregulated compared with cultures supplemented with 0.6% DMSO. The addition of NAC during only the endodermal stage or during the complete hepatocyte differentiation protocol did, however, not improve the generation of hepatocyte-like cells. The redox state of the cell can therefore not explain the effect of DMSO on hepatocyte maturation. In conclusion, addition of DMSO during the hepatocyte differentiation protocol generated a more homogenous population of hepatocyte-like cells but did not induce completely mature hepatocytes.

We previously demonstrated that PSC-derived hepatocyte-like cells support the complete replication of HEV. As DMSO addition improved the homogeneity of hepatocyte-like cells on day 20 of differentiation, we tested if DMSO also rendered the stem PSC-derived hepatocyte-like cells more susceptible for HEV infection. More HEV viral copies were detected, both in the supernatant as well as intracellularly when hPSCs were differentiated using the 0.6-2% DMSO protocol, consistent with the greater homogeneity of the differentiated hepatocyte-like cells. If possible effects of DMSO on the cell membrane, and hence binding of HEV, may play a role in the increased infection efficiency of hepatocyte-like cells will still need to be determined.

Strikingly, but not unexpectedly, 2% DMSO also increased NTCP expression both transcript and protein level. NTCP was recently identified as the key receptor for the entry of the hepatitis B virus into cells (126). In the absence of DMSO, only 3 ± 3.4 % of hepatocyte-like cells expressed NTCP, while treatment with DMSO increased this to 70 ± 5.4 % of hepatocyte-like cells, which was most pronounced in the 0.6-2% DMSO protocol. Moreover, Myrcludex B, a peptide corresponding the pre-S1 domain of HBV was shown to bind to the differentiated hPSC-hepatocyte-like cells (140). Shlomai *et al.* previously demonstrated the presence of HBV pregenomic RNA, cccDNA and HBsAg upon HBV infection of hPSC-HLCs (145). Our preliminary studies confirm these findings and suggest also that PSC-hepatocyte-like cells from DMSO containing cultures might be susceptible to HBV infection, but, additional experiments are necessary to further evaluate the infection of PSC-hepatocyte-like cells with HBV to use this *in vitro* system as a model to evaluate HBV infection.

Chapter V: Hepatocytes: a novel target for Zika virus?

Planning to submit as:

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AUTHOR CONTRIBUTIONS: N.H. and T.T. equally contributed and conceived the study, generated the data and wrote the paper. K.S. contributed to the infection experiments. B.R. helped interpreting results. J.N. and C.M.V. designed and supervised the study.

5.1 Introduction

Zika virus (ZIKV) is a single stranded, positive sense RNA virus, belonging to the *Flaviviridae*. The virus is an arthropod borne virus mostly transmitted by *Aedes* mosquitos. However, cases of sexual transmission and transmission via blood transfusion have as well been described (155, 220, 221). Most ZIKV infected patients are asymptomatic or present only mild clinical symptoms similar to dengue-like illness (222, 223). A major public health concern is however the link between ZIKV infection and abnormalities during fetal brain development. ZIKV viral particles have been detected in the amniotic fluid of pregnant women and in the brain tissue of fetuses with microcephaly (156, 157). Furthermore, the African ZIKV MR766 strain was reported to infect human induced pluripotent stem cell (hiPSC)-derived cortical neural progenitors (NPCs), which resulted in an attenuated growth of the cortical NPCs, due to an increase in apoptosis in the NPC population (160, 162, 163). Recently, mouse models that are susceptible to ZIKV were established. ZIKV infected interferon deficient Ifnar1$^{-/-}$ and AG129 mice contained a high viral load in not only the brain but also in the spinal cord, kidney, spleen, liver and testes (164, 166). Interestingly, a case report published in 1954 described the occurrence of liver damage and jaundice in two patients with elevated ZIKV viral antibodies (224). However, to the best of our knowledge, there is no additional evidence that ZIKV infects hepatocytes and can be a causative agent of hepatitis and/or liver damage.

Dengue virus (DENV) and Yellow Fever virus (YFV), both members of the family of *Flavivirdae* are closely related to ZIKV (225). Both DENV and YFV are known to infect hepatocytes, causing liver damage and jaundice (226-228). Therefore, and based on the finding of high ZIKV load in the liver of murine models, the question arises whether ZIKV may as well be able to infect hepatocytes. However, the availability of primary human hepatocytes (PHHs) is limited and they are known to rapidly dedifferentiate in culture (24, 229). There has been a major interest in the development of functional mature hepatocytes from human pluripotent stem cell (hPSC) to study viral hepatitis, liver disease and liver toxicity *in vitro*. Others and we have previously shown that hPSC-derived hepatocyte-like cells (HLCs) are susceptible to hepatotropic viruses such as the hepatitis E virus (HEV) (144), hepatitis B virus (HBV) (145), hepatitis C virus (HCV)

and dengue virus infection (143, 190, 192, 230). hPSC-derived hepatocytes are a valuable and physiologically relevant model to study the tissue tropism of viruses and to examine the host response induced upon infection.

We here demonstrate that both human embryonic (hESC)- and hiPSC-derived hepatocyte-like cells (HLCs) support the complete replication cycle of ZIKV including entry, replication and the production of infectious virions, while infection was inhibited by 7-deaza-2'-*C*-methyladenosine (7DMA) treatment. In addition, ZIKV induced a host response in PSC-HLCs, which was absent in an infected hepatoma cell line. These results indicate that ZIKV can target hepatocytes and might like other *Flaviviridae* cause liver damage.

5.2 Materials and methods

5.2.1 Cell cultures. The hESC line H9 was purchased from WiCell Research Institute (Madison, US). The fibroblast cell line BJ1 was previously reprogrammed to hiPSCs using lentiviral transduction of OCT4, SOX2, KLF4 and c-MYC and was fully characterized as described (21). A second hiPSC line was obtained from Sigma-Aldrich (Saint Louis, MO). hPSCs were maintained on human matrigel (BD Biosciences, San Jose, CA) coated plates in E8 Flex medium (Gibco) in a humidified 5% CO2 incubator at 37°C. hPSC were differentiated towards HLCs following a previously published 20-day differentiation protocol with minor adjustments (21,26). Dimethylsulfoxide (DMSO) in a concentration of 0.6% was added during differentiation from day 0 until day 12 and increased to 2% from day 12 until the end of the hepatocyte differentiation protocol. The human hepatoma cell line HuH7 (kindly provided by Ralf Bartenschlager, University of Heidelberg, Germany) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% non-essential amino acids (Gibco) and 1% penicillin-streptomycine in a humidified 5% CO2 incubator at 37°C.

5.2.2 Virus. Infections were performed using the ZIKV strain MR766 (Rhesus/1947/Uganda) and the NR-50240 strain (clinical isolate) (PRVABC59) (Human/2015/Puerto Rico). Viral stocks were generated as previously described (13).

5.2.3 Virus inoculation. On day 16 of differentiation, hPSC-derived HLCs were inoculated with 2% ZIKV stock (viral titer: $1x10^3$ PFU/ml) in hepatocyte differentiation medium and incubated during 6h at 37°C in a 5% CO_2 humidified incubator. Following the 6h incubation, the cells were washed extensively with PBS and fresh hepatocyte differentiation medium was added. Half of the medium was replaced every other day and supernatant was collected 2, 4 and 6 days after infection. Cells were lysed with 350ul RLT buffer 4 and 6 days after infection (Qiagen, Hilden). Infection of Huh7 cells was performed similarly.

5.2.4 Inhibition experiments. 7-deaza-2'-C-methyl-D-adenosine (7DMA) and 2'-C-methylcytidine (2'CMC) were purchased from Carbosynth (Berkshire, UK). T705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide; Favipiravir) was obtained as custom synthesis product from abcr GmbH (Karlsruhe, Germany). ZIKV infected cells were treated with 3 different concentrations of each compound (7DMA: 10, 30 and 90μM; 2'CMC: 5, 15 and 45μM ; T705: 25, 75 and 225μM) based on the EC_{50} using Vero cells (African Green monkey kidney cells; ECACC) as previously published (13). 7DMA and 2'CMC were added to the medium at the time of the viral inoculation until the end of the infection experiment, while T705 was added 1h before inoculation with ZIKV.

5.2.5 RNA isolation and quantitative RT-qPCR. Viral RNA was extracted from 150ul supernatant using the Nucleospin RNA cirus kit (Macherey-Nagel, Düren) following the manufacturer's protocol. Intracellular RNA was isolated by means of the RNeasy Mini kit (Qiagen). RT-qPCR was performed to analyze viral RNA levels as previously described (13). Detected viral RNA levels in the lysates were normalized for total RNA content. Total RNA content was measured by spectrometry (Nanodrop ND-1000, Thermo Fischer Scientific). For gene expression analysis, cDNA synthesis was performed using the Superscript III First-Strand synthesis kit (Invitrogen). Following cDNA synthesis, RT-qPCR was performed with the Platinum SYBR green qPCR supermix-UDG kit (Invitrogen) using the ViiA7 Real-Time PCR instrument (Thermo Fischer Scientific, Waltham, US). The housekeeping gene glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was used for normalization. All qRT-qPCR primer sequences are listed in (Table 6).

Gene	Forward	Reverse
GAPDH	TCAAGAAGGTGGTGAAGCAGG	ACCAGGAAATGAGCTTGACAAA
AFP	TGAGCACTGTTGCAGAGGAG	GTGGTCAGTTTGCAGCATTC
ALB	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA
AAT	AGGGCCTGAAGCTAGTGGAT	TCCTCGGTGTCCTTGACTTC
HNF4α	ACTACGGTGCCTCGAGCTGT	GGCACTGGTTCCTCTTGTCT
NTCP	ATCGTCCTCAAATCCAAACG	CCACATTGATGGCAGAGAGA
EIF2AK2	GAGAATTTCCAGAAGGTGAAGGT	ATTCCCCATGGATAATCCTTCT
MX1	TGCTTATCCGTTAGCCGTGG	CGCCAGCTCATGTGCATCT
ISG15	GAGAGGCAGCGAACTCATCT	CTTCAGCTCTGACACCGACA
IFNβ	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG
CXCL2	CCACACTCAAGAATGGGCA	CAATAAGCTTCCTCCTTCCTTCTG
CXCL3	TAGCCACACTCAAGAATGGGAA	TCTCTCCTGTCAGTTGGTGC
NFκB	CAGAGAGTGAGGATGAGGAGAG	TCATCATAGGGCAGCTCGT

Table 6. *Primers used for RT-qPCR.*

5.2.6 Immunofluorescence. Cells were fixed with 4% paraformaldehyde (PFA) 4 days after infection and washed with PBS. Cells were permeabilized with 0,2% Triton-X in PBS and blocked with 5 % donkey serum in PBS. The cells were stained overnight at 4°C with 1:2000 rabbit anti-HNF4α (Ab41898, Abcam), 1:500 mouse anti-Flavivirus Group Antigen (clone D1-4G2-4-1, Millipore) and/or 1:200 rabbit anti-caspase 3 (active, cleaved form) (AB3623, Millipore) antibody. Afterwards, the cells were incubated for 1h at room temperature with the appropriate secondary antibodies and Hoechst (Sigma-Aldrich). Appropiate isotye control antibodies were used for all images. Images were taken using the AxioimagerZ.1 fluorescence microscope.

5.2.7 Re-infection assays. hESC-HLCs were inoculated with 6 days post infection supernatant of ZIKV infected hESC-HLCs (1:10 dilution). After incubation for 6h at 37°C in a 5% CO_2 humidified incubator, the inoculum was removed and the cells were washed extensively with PBS. Supernatant was collected 2 and 4 after post infection. At day 4 after infection, cells were lysed with 350 µl RLT buffer (Qiagen).

5.2.8 MTS assay. Cells were seeded and differentiated in a 96-well format plates. On 6 days after infection, the medium was removed from the ZIKV infected cells and 100 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazinemethosulfate (MTS/PMS; Promega, Leiden, Netherlands) was added. After 1.5h incubation at 37°C in a 5% CO_2 humidified incubator, the optical density (OD) was determined at 498 nm using the Tecan Safire^{2} ™. The % cell survival was calculated using the following formula: % survival = 100 x (OD_{IC}/OD_{UIC}).

5.2.9 Statistics. Data is represented as mean ± SEM and analyzed by the two-tailed Student's t test. p-values < 0.05 (*)(+)(#), p < 0.01 (**)(++)(##), p < 0.001 (***)(+++)(###) and p < 0.0001 (****)(++++)(####) were considered statistically significant.

5.3 Results

5.3.1 Hepatocyte-like cells and hepatoma cells are susceptible to ZIKV

hESCs and hiPSCs were differentiated toward hepatocyte-like cells (HLCs). Both, day 16 differentiated HLCs and the HuH7 hepatoma cell line were infected with the African MR766 ZIKV strain. Zika viral RNA levels were quantified by RT-qPCR on the supernatant on day 2, 4 and 6 after infection (days post infection, dpi) and in the cellular lysates (intracellular) 4 and 6 days after infection. Infection efficiency was comparable between ZIKV infected hPSCs-derived HLCs and HuH7 cells with approximately 1 x 10^{8} viral RNA copies mL^{-1} or μg^{-1} in the supernatant or cellular lysates, respectively (Figure 30A). Four days after infection, ZIKV infection of hPSC-HLCs and HuH7 cells was confirmed by immunofluorescence staining demonstrating 10 ± 2.1% and 12.4 ± 2.0% ZIKV infected hPSC-HLCs and HuH7 cells, respectively. In addition, ZIKV infected hPSC-HLCs and HuH7 cells were positive for the liver-enriched transcription factor HNF4α (Figure 30B).

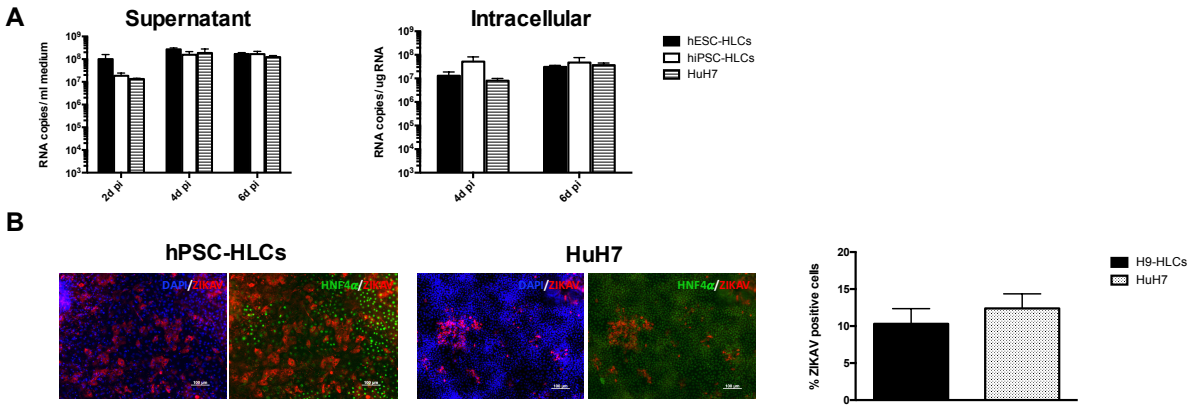

Figure 30. *Infection of hPSC-HLCs and HuH7 with MR766 zika viral strain. (A) RT-qPCR was used to quantify the viral RNA levels in the supernatant and intracellularly (d pi = days post infection) (n=3). (B) Left; Immunofluorescence staining for HNF4α and ZIKV in infected hPSC-HLCs and HuH7 cells 4 days after infection. Right; quantification of immunofluorescence staining. Images are representative of three independent experiments (Scale bar = 100μM).*

5.3.2 Differences in inhibition of ZIKV replication in HLCs and HuH7 cells

Zmurko *et al.*, recently demonstrated the antiviral activity of three viral RNA-dependent RNA polymerase inhibitors: 2'-*C*-methylcytidine, 7-deaza-2'-*C*-methyladenosine (7DMA), 2'-*C*-methylcytidine (2' CMC) and 6-fluoro-3-hydroxy-2-pyrazinecarboxamide (Favipiravir; T705), on ZIKV infected Vero cells (164). The administration of 7DMA (10μM - 90μM) significantly inhibited ZIKV replication in a dose-dependent manner in the cellular lysates and supernatants of both, ZIKV infected hPSC-HLCs and HuH7 cells (Figure 31). However, even at the highest concentration used, 7DMA did however not completely abolish ZIKV replication as ZIKV RNA levels continued to increase over time (Figure 31). 2'CMC and T705 treatment significantly inhibited viral replication in HuH7 infected cells but failed to inhibit ZIKV replication in hPSC-HLCs (Figure 32).

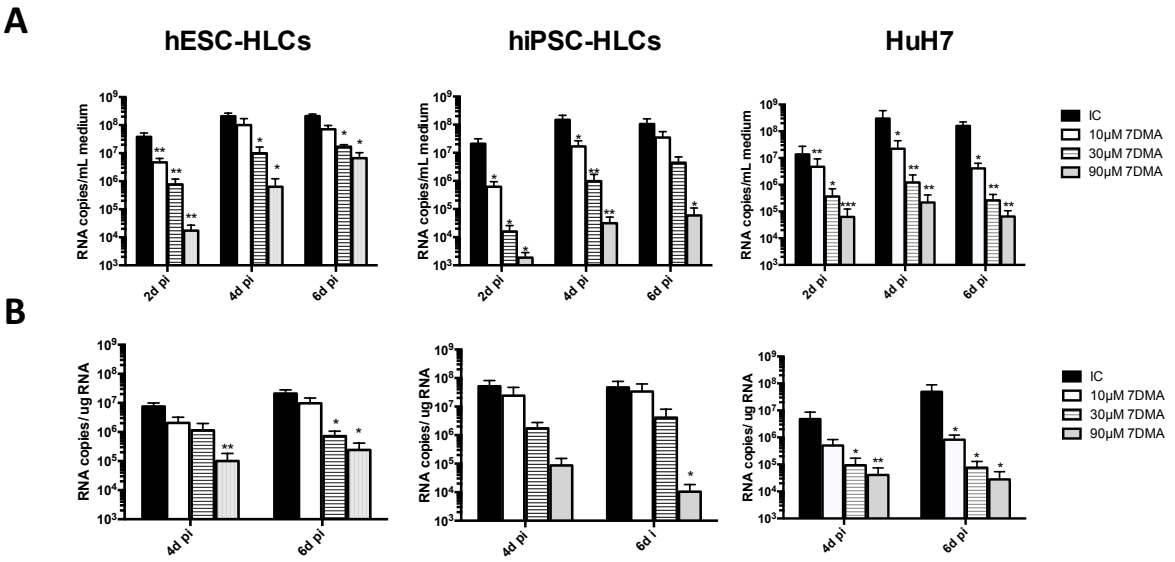

Figure 31. *Inhibition of ZIKV replication by 7DMA. (A) RT-qPCR analysis of the supernatant of ZIKV infected hESC-HLCs, hiPSC-HLCs and HuH7 cells. ZIKV replication was inhibited by increasing concentrations of 7DMA (10μM - 90μM) (IC = infected cell) (n=3; *: p=0.05). (B) RT-qPCR analysis of the cellular lysates of ZIKV infected hESC-HLCs, hiPSC-HLCs and HuH7 cells. ZIKV replication was inhibited by increasing concentrations of 7DMA (10μM - 90μM) (n=3; *: p=0.05).*

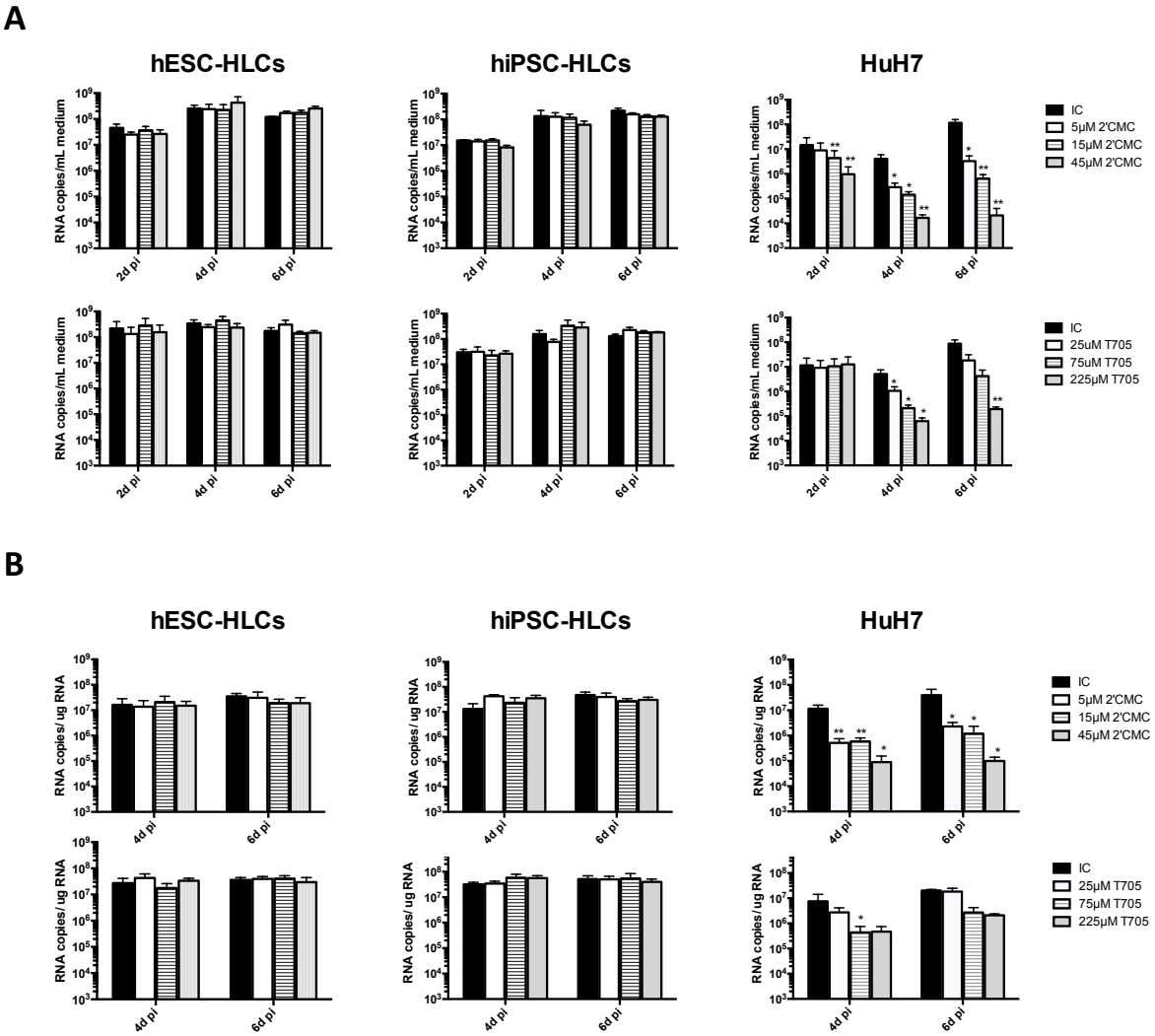

Figure 32. *Inhibition of ZIKV replication by 2'CMC and T705. (A) RT-qPCR analysis of the supernatant of ZIKV infected hESC-HLCs, hiPSC-HLCs and HuH7 cells. Infected cells (IC) were treated with increasing concentrations of 2'CMC (5μM - 45μM) or T705 (25μM - 225μM) (n=3; *: p=0.05). (B) RT-qPCR analysis of the cellular lysates of ZIKV infected hESC-HLCs, hiPSC-HLCs and HuH7 cells. Infected cells were treated with increasing concentrations of 2'CMC (5μM - 45μM) or T705 (25μM - 225μM) (n=3; *: p=0.05).*

5.3.3 ZIKV infected hPSC-HLCs produce infectious virions

The supernatant collected from ZIKV infected hPSC-HLCs was used to re-infect hPSC-HLCs. Infection was quantified by RT-qPCR and viral RNA was detected 2 and 4 days after infection in the supernatant and as well 4 days after infection in the cellular lysates of re-infected hPSC-HLCs. To examine the efficiency of 7DMA treatment, we also

collected the supernatants (SN) of 7DMA treated primary-infected hPSC-HLCs and used this for re-infection experiments. Although 7DMA significantly inhibited ZIKV replication (Figure 31), ZIKV persisted in sufficient levels to allow re-infection of secondary PSC-HLCs. Although re-infection of hPSC-HLCs with supernatant collected from cells treated with either 30μM or 90μM 7DMA was significantly lower on day 2 compared to cells re-infected with supernatant from initial cultures not treated with 7DMA, viral RNA levels on day 4 either in the supernatant or cellular lysates of secondarily infected hPSC-HLcs was not significant different when supernatants from 7DMA treated or untreated (IC) primary infected hPSC-HLCs were used (Figure 33).

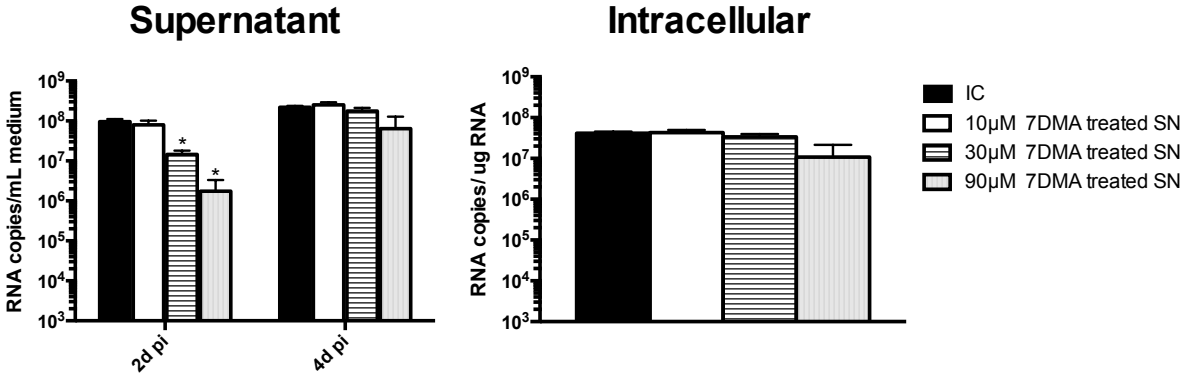

Figure 33. *ZIKV infected hPSC-HLCs produce infectious virions. hPSC-HLCs were infected with supernatant (SN) of ZIKV infected but untreated hPSC-HLCs (IC) or with supernatant (SN) of ZIKV infected but 7DMA treated hPSC-HLCs. RT-qPCR was used to quantify ZIKV RNA (n=3; *: p=0.05).*

5.3.3 ZIKV induces a cytopathic effect

ZIKV infection of hPSC-HLCs and HuH7 cells induced a cytopathic effect (CPE). CPE was assessed 6 after post infection by microscopic evaluation, MTS readout assay and active Caspase-3 staining. The survival rate of ZIKV infected hPSC-HLCs was approximately 80%. Although the infection efficiency of hPSC-HLCs and HuH7 was comparable (Figure 30), ZIKV infected HuH7 had a survival rate of only 31.6 ± 4.09%. CPE was significantly reduced in ZIKV infected hPSC-HLCs that were treated with increasing concentrations of 7DMA. Treatment of ZIKV infected hPSC-HLCs with 2'CMC or T705 did not reduce CPE, in line with our observation that 2'CMC or T705 did not reduce ZIKV RNA levels in

infected hPSC-HLCs. By contrast, 7DMA, 2'CMC and T705 significantly reduced CPE in ZIKV infected HuH7 cells, consistent with the viral RT-qPCR data. In addition, ZIKV infected hPSC-HLCs stained positive for activated Caspase-3 (Figure 34B).

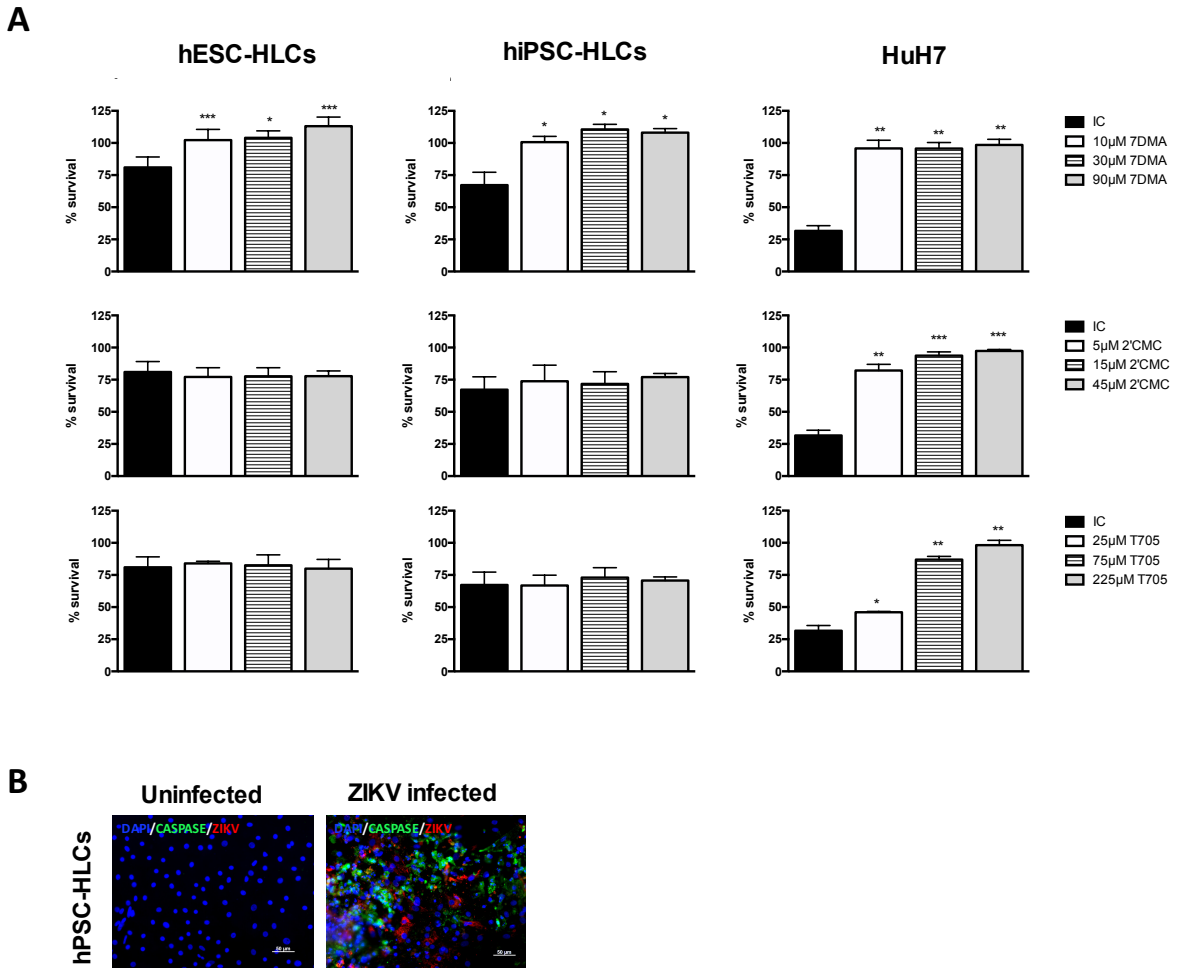

Figure 34. *ZIKV induces CPE. (A) CPE was quantified by MTS readout assay. Cells were either untreated (IC = infected cell) or treated with 7DMA, 2'CMC or T705 (n=3; *p=0.05%). (B) Immunofluorescence staining for activated Caspase-3. Images are representative of three independent experiments (Scale bar = 50μM).*

5.3.4 ZIKV induces a host immune response in hPSC-HLCs but not in HuH7 cells

Upon viral infection, the innate immune system of cells is triggered to clear the virus from the infected cells. Therefore, we quantified transcript levels of the interferon stimulated genes (ISGs) *EIF2AK2, MX1, IFNβ* and *ISG15* by RT-qPCR in ZIKV infected

cells. All ISGs were significantly more upregulated in both ZIKV infected hESC- and hiPSC-HLCs compared to ZIKV infected HuH7 cells. The expression of all ISGs was significantly and dose-dependently downregulated upon 7DMA treatment of ZIKV infected hPSC-HLCs. There was no effect on the expression of ISGs when infected hPSC-HLCs were treated with either 2'CMC or T705 consistent with our observation that these two antivirals failed to inhibit ZIKV replication in hPSC-HLCs. Although 7DMA, 2'CMC and T705 all inhibited viral replication in HuH7 cells, treatment with these compounds had no significant effect on the expression of ISGs (Figure 35).

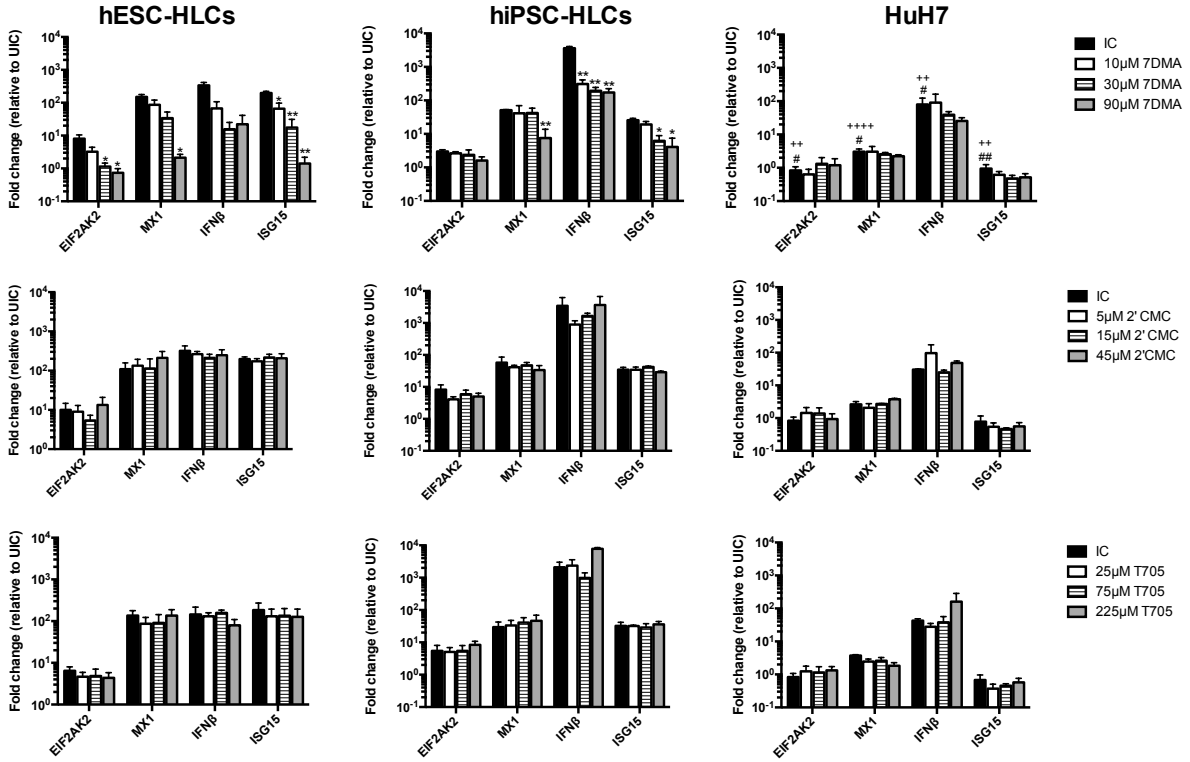

Figure 35. *ZIKV induces an innate immune response only in infected hPSC-HLCs. RT-qPCR analysis for different ISGs. (IC = infected cell) (n=3; * significance of treated cells to IC; + significance of IC HuH7 to IC hESC-HLCs; # significance of IC HuH7 to hiPSC-HLCs).*

As ZIKV induced an interferon immune response and ZIKV infected cells rapidly underwent apoptosis, we examined the induction of the NFκB stress response upon infection. Similar to the induction of ISGs, transcripts for *NFκB* and its target genes *CXCL2* and *CXLC3,* were significantly more induced in ZIKV infected hPSC-HLCs compared to ZIKV infected HuH7 cells, even if the latter were more prone to ZIKV mediated CPE. Inhibition of ZIKV replication by 7DMA in infected HLCs significantly

decreased the transcript levels of *NFκB* and its target genes. In HuH7 cells, the NFκB host response was not influenced by the antiviral compounds. (Figure 36).

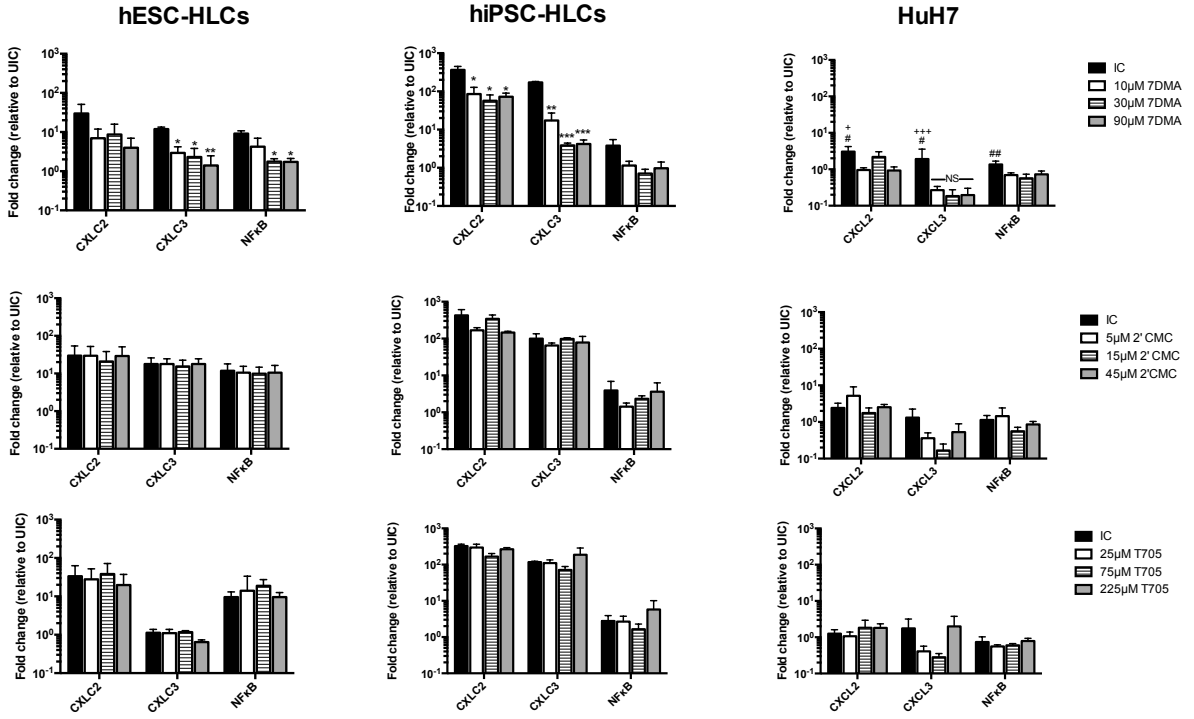

Figure 36. ZIKV *induces an NFκB response only in infected hPSC-HLCs. RT-qPCR analysis for NFκB, CXLC2 and CXCL3. (IC = infected cell) (n=3; * significance of treated cells to IC; + significance of IC HuH7 to IC hESC-HLCs; # significance of IC HuH7 to hiPSC-HLCs).*

5.3.5 Infection of hPSC-HLCs with the Asian ZIKV lineage

We demonstrated that hPSC-HLCs were as well susceptible to infection with a ZIKV clinical isolate from the Asian lineage. hPSC-HLCs were infected with the same viral titer as the MR766 African strain. The Asian strain induced significantly more CPE compared to the African strain (40.9% vs. 81.0% survival). Presence of the Asian lineage of ZIKV in infected hPSC-HLCs was further demonstrated by immunofluorescence, even if quantification of the percentage ZIKV infected HLCs was difficult due to the high levels of apoptotic cells. 7DMA also inhibited replication of the Asian strain of ZIKV demonstrated by a significant CPE reduction.

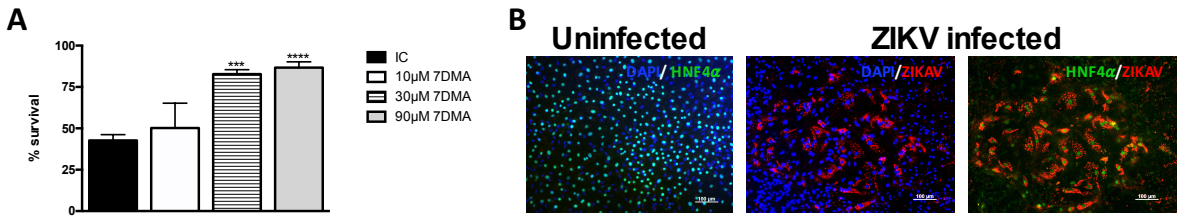

Figure 37. *Infection of hPSC-HLCs with the Asian ZIKV strain. (A) MTS assay was used to quantify the % survival upon ZIKV infection. Infected cells were either untreated (IC) or treated with increasing concentration of 7DMA (n=3; *p=0.05%). (B) The presence of ZIKV was as well confirmed by immunofluorescence staining. Images are representative of three independent experiments (Scale bar = 100μM).*

5.4 Discussion

The fact that hPSCs can be differentiated in all cells of the human body, make them an interesting tool to study the tissue tropism of viruses. Currently, knowledge regarding the tissue tropism of ZIKV is very limited. Recent studies demonstrated that hPSC-derived neuroprogenitors are susceptible to ZIKV *in vitro* (160, 162, 163). In addition, studies wherein interferon-deficient mouse models were infected with ZIKV have suggested that high viral titers of ZIKV can be detected not only in the brain but also in the testes, spleen, kidney and liver (164, 165). ZIKV belongs to the virus family of *Flaviviridae*, which also includes dengue, yellow fever and hepatitis C virus, all known to infect hepatocytes and cause acute hepatitis (152, 227). A case report published in 1954 suggested that tow patients with ZIKV antibodies also had signs of hepatitis, including jaundice (224). However, aside from the mouse model and these case reports, there are to the best of our knowledge, no cases of liver damage reported following ZIKV infection. Nevertheless, in view of the data from the mouse models and the fact that ZIKV belongs to a strain of viruses known to have hepatocyte tropism and cause acute hepatocyte damage, we here examined if human hepatocytes can be infected by ZIKV.

Therefore, we infected hPSC-HLCs and HuH7 cells with the ZIKV MR766 African strain. Both, hESC and hiPSC-HLCs as well as HuH7 cells could be infected with ZIKV as was demonstrated by quantification of ZIKV RNA, ZIKV immunofluorescence staining and the fact that of infectious virions were produced, that could re-infect other hPSC-HLCs. We also demonstrated that hPSC-HLCs were susceptible to infection with a

clinical isolate of the Asian type ZIKV. The Asian type ZIKV is responsible for the recent outbreaks of ZIKV in Latin-America and is believed to be associated with an increased risk for microcephaly in newborns (156, 231). Our studies suggest that the Asian type ZIKV strain may also be more efficacious in infecting hPSC-HLCs, as hPSC-HLC CPE caused by the Asian strain ZIKV at the same MOI as used for the African strain was significantly higher.

A recent study has demonstrated that 7DMA can delay the disease progression in a ZIKV infected mice (164). We here demonstrated that 7DMA could as well inhibit viral replication of both, the African and Asian ZIKV strain in a dose-dependent way in infected HuH7 cells and hPSC-HLCs. By contrast, 2'CMC and T705, previously shown to inhibit ZIKV in Vero cells (164), inhibited ZIKV replication only in HuH7 cells. However, even the highest concentration of 7DMA used could not completely clear ZIKV from infected hPSC-HLCs, as supernatants from 7DMA treated ZIKV infected hPSC-HLCs still contained infectious virions that could re-infect secondary hPSC-HLCs cultures.

As is also seen for other *Flavivirdae*, ZIKV infection caused CPE in both, ZIKV infected HuH7 cells and hPSC-HLCs. Although the percentage of ZIKV infected cells was comparable between HuH7 cells and hPSC-HLCs, CPE was more pronounced in infected HuH7 cells. Consistent with the viral transcript studies, CPE was completely abolished in HuH7 cells and hPSC-HLCs by 7DMA, while 2'CMC and T705 only reduced CPE in ZIKV infected HuH7 cells. Although autophagy usually kills pathogens, flaviviruses such as dengue virus were shown to modulate autophagy-dependent processing of lipid droplets to benefit their replication. Liang *et al*. recently demonstrated that ZIKV non-structural proteins NS4A and NS4B also induced autophagy to promote its replication which eventually leads to cellular dysregulation, attenuated growth and cell death (232).

Hamel *et al.*, demonstrated the induction of interferon stimulated genes (ISGs) is triggered upon ZIKV infection of human dermal fibroblasts and epidermal keratinocytes (159). Moreover, only interferon deficient mice were found to be permissive to ZIKV and the virus was recently demonstrated to evoke a human innate immune response through degradation of STAT2, which is a transcriptional activator of interferon stimulated genes (ISGs) (164, 165, 233). Consistent with these *in vitro* and *in vivo* published results, we demonstrated that ZIKV induced ISGs significantly in hPSC-HLCs. Interestingly, this was not the case in ZIKV infected HuH7 cells. Consistently, we found

induction of *NFκB* and target genes only in hPSC-HLCs and not HuH7 cells, even if the latter displayed more extensive CPE following ZIKV infection.

In conclusion, hepatoma cells as well as hPSC-HLCs are susceptible to ZIKV infection. The antiviral effect of three viral polymerase inhibitors and the host response upon infection were however significantly different between ZIKV infected HuH7 cells and hiPSC-HLCs. We believe that hPSC-HLCs more closely resemble primary human hepatocytes (PHH) and better recapitulate the *in vivo* situation. However, ZIKV infection studies with PHH are necessary to confirm our findings. As hPSC-HLCs mimic fetal rather than mature hepatocytes (56, 57), we hypothesize that ZIKV might infect as well fetal liver but additional studies are required. We further demonstrated that the host response and the effect of antiviral compounds are highly cell type- and cell line-dependent. It is crucial to validate the effect of antiviral drugs not only in cancerous (e.g. hepatoma cell lines) and immortalized (e.g. Vero cells) cell lines but also in more physiologically relevant systems such as differentiated hPSC and primary human cultures.

Chapter VI: General conclusion

6.1 Conclusion

The study of hepatotropic viruses is hampered by to the lack of efficient and physiologically relevant cell culture and animal models. Primary human hepatocytes (PHH) are the gold standard to examine the replication of hepatotropic viruses and inhibition thereof. However, PHH are scarce and highly variable among different donors. Moreover, cultured PHH dedifferentiate in less than 24 hours and lose their phenotypic characteristics, including their main drug metabolizing enzymes.

hPSC-derived hepatocyte-like cells are a valuable alternative to PHH as hPSCs have the ability to indefinitely self-renew and to differentiate into all cell types of the human body. In addition, the iPSC technology allows to create patient-specific models and overcomes the limitation of donor variability and shortage. However, to date and to the best of our knowledge, no research group succeeded in the generation of fully mature hepatocytes. Compared to PHH, hPSC-derived hepatocyte-like cells (hPSC-HLCs) are immature and characterized by persistent alpha-fetoprotein (*AFP*) expression and low metabolizing enzyme expression and function (56, 57). In accordance, the hepatocyte differentiation protocol published by the Verfaillie lab is robust but generates HLCs that are unfortunately not completely mature yet (169). Nevertheless, Roelandt *et al.,* among others, published in 2012 that hPSC-derived HLCs are susceptible to HCV infection (143).

In this thesis, I demonstrated that hPSC-derived HLCs also support the complete replication cycle of the hepatitis E virus (HEV). HEV infection was demonstrated by the presence of (+) and (-) ssRNA as quantified by RT-qPCR. In addition, viral replication was inhibited by ribavirin and interferon-alpha 2b treatment. Interestingly, we showed that neuroprogenitors and mesodermal cells, both derived from hESCs, did only support HEV replication when the virus was transfected in the cells. We therefore hypothesize the presence of a hepatocyte-specific receptor, which is absent on hPSC-derived neuroprogenitor and mesodermal cells and is required for entry of HEV in hepatocytes, the nature of which is yet to be discovered. hPSC-HLCs are therefore a valuable alternative to hepatoma cell lines and PHH to study the biology of HEV and might aid in the identification of the HEV receptor(s) (144).

Secondly, this thesis assessed means to improve the hepatocyte differentiation from hPSCs by the addition of dimethyl sulfoxide (DMSO). DMSO was previously shown to prevent too some extent the dedifferentiation of cultured PHH and to induce differentiation of the bipotential hepatoma cell line, HepaRG (27, 206). The addition of 0.6% DMSO significantly improved endoderm formation which was confirmed by RT-qPCR, SOX17 staining and CXCR4/cKIT flow cytometry, resulting in a more homogenous population of hPSC-derived endodermal cells. By comparing different concentrations of DMSO, we concluded that a concentration of 0.6% DMSO between day 0 and day 12 followed by an increased concentration of 2% from day 12 onwards generated a more homogenous population of hPSC-HLCs that expressed significantly more *AAT, HNF4α* and *NTCP* transcripts and proteins. I hypothesized that the effect of DMSO could be due to its antioxidant and free radical scavenging properties. To confirm this hypothesis, I examined the effect of a well-known antioxidant, N-acetylcysteine (NAC), on endoderm and subsequent hepatocyte differentiation. Addition of NAC significantly improved definitive endoderm formation but did, however, not improve hepatocyte differentiation/maturation. The mechanism of action of DMSO and NAC might therefore be different and not be regulated by the induction or reduction of ROS. In addition, ROS assays failed to detect increased or reduced ROS levels in DMSO or NAC – treated cells. Moreover, DMSO is also known to have oxidizing properties and increased ROS levels are believed to induce stem cell differentiation (234). Our results are therefore conflicting and additional experiments will be needed to elucidate the role of DMSO and NAC during stem cell differentiation. What was however striking was the tremendous upregulation of NTCP in DMSO treated hPSC-HLCs. As NTCP was recently identified as the key receptor for HBV entry (180), I examined whether hPSC-derived HLCs also supported the replication of HBV. I first demonstrated binding of Myrcludex B, a peptide corresponding the pre-S1 domain of HBV to hPSC-derived HLCs. In addition, I showed the presence of HBcAg by immunofluorescence staining. However, these results are preliminary and should be further confirmed by additional assays.

Finally, I demonstrated that hPSC-HLCs are also susceptible to ZIKV infection. ZIKV recently gained ample attention due to major outbreaks in Latin-America, and the possible link between ZIKV infection and microcephaly in newborns during this outbreak (156, 157). ZIKV infected mouse models have been established that sustained high viral load not only in the brain but also in the liver (164, 165). As ZIKV is closely

related to dengue and yellow fever virus, both known to infect and replicate in hepatocytes (226, 228), I tested if ZIKV would also infect hPSC-HLCs. I first demonstrated by RT-qPCR and immunofluorescence that the African MR766 ZIKV strain infected both hPSC-HLCs and the hepatoma cell line, HuH7. Infection was dose-dependently inhibited in both cell types by treatment with the viral polymerase inhibitor 7DMA. Two other viral polymerase inhibitors, 2'CMC and T705 inhibited ZIKV infection in HuH7 cells but not in ZIKV infected hPSC-HLCs. A host innate immune and NFκB response were induced in ZIKV infected hPSC-HLCs but not in infected HuH7 cells. Finally, a clinical isolate from the ZIKV Asian strain, which caused the recent outbreak in Latin-America, was also shown to infect hPSC-HLCs and HuH7 cells. Although there are, to the best of our knowledge, no clinical data about ZIKV induced liver damage or hepatitis, hPSC-HLCs may be a good model to study ZIKV infection, including the assessment of the host response, which was only induced upon infection of hPSC-HLCs but not upon infection HuH7 cells. Moreover, the antiviral drug activity was highly dependent on the cell type used.

In conclusion, I demonstrated that hPSC-HLCs are a valuable alternative to hepatoma cell lines and PHH to study the biology of replication of hepatotropic viruses. In addition, the differentiation capacity of hPSCs allows to generate from a single cell population all cell types of the human body representing an interesting tool to study the host and tissue tropism of different viruses.

6.2 Future perspectives

Our studies demonstrated the susceptibility of hPSC-derived HLCs to different hepatotropic viruses. hPSC-derived HLCs are susceptible to HCV, HEV, HBV and ZIKV and provide a tool to study the biology of replication of these viruses and to test the efficiency of antiviral drugs. Future studies should however still be performed to: (i) improve the maturation of hPSC-HLCs, (ii) identify the hepatocyte-specific HEV receptor, (iii) study the infection of hPSC-HLCs with HBV and (iv) study *in vivo* the biology of replication of hepatotropic viruses in hPSC-derived humanized mouse models.

(i) Maturation of hPSC-HLCs

Many research groups have succeeded in the differentiation of PSCs toward HLCs (47, 50, 168, 169, 207). However, all studies demonstrate that only immature hepatocytes can be generated that resemble fetal hepatocytes and also display some of the features of de-differentiated PHHs (56, 57). As some important drug metabolizing enzymes, such as CYP3A4, are only poorly expressed in the differentiated PSC progeny, the use of PSC-HLCs to test antivirals remains limited. In addition, hepatocytes are not only responsible for drug inactivation but also for drug activation. Many antivirals are synthetized as prodrugs and a physiological relevant drug metabolizing machinery is therefore inevitable to assess their efficacy. The process of hepatocyte maturation remains to be elucidated. Recent advances using fir instance overexpression of key hepatocyte TFs and improved cell culture techniques are promising, but still have not yet allowed generation of mature hPSC-hepatocytes. As transcription factors and microRNAs (miRNAs) are known to be key regulators of gene expression, activation and inactivation of transcription factors and miRNAs that are not sufficiently expressed or are miss-expressed, respectively, (57) by novel genome editing techniques such as CRISP/Cas9 might induce hepatocyte maturation. In addition, using culture systems reflecting the physical properties of liver, most importantly the low stiffness (4 Kilo Pascal) as well as liver sinusoid specific extracellular matrix components, and 3D co-culture systems of hepatocyte with ideally stem cell-derived non-parenchymal cells such as LSECs will be necessary to better recapitulate the liver niche in a dish.

(ii) Identify HEV receptor(s)

One of the advantages of hPSCs is their multi-lineage differentiation potential. We demonstrated that hPSCs can be differentiated to hepatocytes, mesodermal cells and neuroprogenitors, among others. Only hPSC-derived hepatocytes were susceptible to HEV infection. However, mesodermal cells and neuroprogenitors do support HEV replication when HEV was transfected in the cells. We therefore hypothesize that hPSC-derived mesodermal and neuroprogenitor cells lack a hepatocyte-specific receptor (144). Membrane proteomics, microarray and/or RNA-sequencing studies may uncover the identity of differentially expressed proteins and genes between these three cell populations and aid in the identification of one or more HEV candidate receptor(s).

Identification of the HEV receptor might be a potential candidate to inhibit viral entry and subsequent HEV infection, as has been recently shown for HBV.

(iii) Study the infection of hPSC-HLCs with HBV

As proof of principle, we demonstrated by HBcAg staining that hPSC-HLCs are susceptible to HBV infection. Additional experiments need to be performed to demonstrate the suitability of this model to study the biology of HBV replication and inhibition thereof. HBV infection should be monitored by means of measuring secreted HBcAg by ELISA. Detection of pregenomic RNA, which is not present in the particle and thus not in the inocula, should be quantified by RT-qPCR to detect active viral replication. Scaled up differentiations should then be used to study the production of HBV cccDNA and pregenomic RNA synthesis by Southern and Northern hybridization, respectively. In addition, the antiviral effect of HBV inhibitors such as Myrcludex B and tenofovir could then be evaluated using the aforementioned methods. If hPSC-HLCs are susceptible to HBV infection and the deposition of cccDNA, I hypothesize that as PSc0HLCs can be maintained for at least another 10 days following differentiation to the HLC as described in this thesis, the model could be used to perform high throughput antiviral drug screens to eliminate cccDNA, and to study the host response following HBV infection.

(iv) Humanized mouse models

To recapitulate and predict the complex process of absorption, distribution, metabolism and excretion (ADME), relevant *in vivo* models are necessary. Two frequently used humanized liver mouse models are the urokinase plasminogen activator (uPA) and the fumaryl acetoacetate hydrolase (FAH) mouse model. In these mouse models, liver damage is caused by hepatocyte specific overexpression of uPA or by loss of FAH, respectively. Both mouse models can be back-crossed to mice with an immunodeficient phenotype, permitting robust engraftment of PHH (118, 147). Humanized mouse models can more closely predict the pharmacokinetics, pharmacodynamics and metabolism of drugs than *in vitro* models. Humanized mouse models are also useful to model infectious diseases caused by hepatotropic viruses such as HBV, HCV and HEV, which display a unique human hepatocyte tropism, and other infections such as by *Plasmodium* parasites (117, 118, 235). hPSC-derived hepatocytes would be an ideal

alternative source of hepatocytes, because they are unlimited in supply, and could be created from a diverse panel of donors with different susceptibility to drug toxicity and viral infections. Unfortunately, engraftment of the immature hPSC-HLCs is not robust (236). Increased maturation of hPSC-HLCs will be crucial to allow them to efficiently repopulate damaged mouse livers. Another drawback is that the immune responses cannot be studied in these immunodeficient mouse models. Theoretically, it might be possible to create mouse models that harbor aside from human hepatocytes as well as an HLA-identical humanized immune system (146), both derived from PSCs, which would provide an ideal model to study hepatitis virus pathology and identify efficient antiviral approaches for hepatotropic pathogens.

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Curriculum Vitae

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Education

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September 2010 – June 2012 Master in Pharmaceutical sciences magna cum laude at the Catholic University of Leuven, Belgium

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Research Experience

August 2012 – present PhD thesis: Infection of stem cell-derived hepatocytes with hepatotropic viruses.

February 2011 – June 2012 Master's degree experimental thesis: Characterization of RNA aptamers targeting tPA.

February 2011 – June 2011 Erasmus student in the Department of Molecular Biology under supervision of Professor Peter André Andreasen at Arhus Univeristy, Denmark

Publications

Jolien Vanhove, Mariaelena Pistoni, Marc Welters, Kristel Eggermont, Veerle Vanslembrouck, **Nicky Helsen**, Ruben Boon, Mustapha Najimi, Etienne Sokal, Philippe Collas, J. Willem Voncken, Catherine M. Verfaillie. H3K27me3 does not orchestrate the expression of lineage-specific markers in hESC-derived hepatocytes in vitro. Stem cell reports. 2016;7(2):192-206.

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Other publications

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Poster presentations

2nd Annual meeting of the Belgian Society for Stem Cell Research (BeSSCR), 18 September 2015, Brussels, Belgium. Improvement of definitive endoderm formation by targeting the redox state. (Poster presentation).

Annual meeting International Society for Stem Cell Research (ISSCR), 24-27 June 2015, Stockholm, Sweden. Infection of stem cell-derived hepatocytes with the hepatitis E virus. (Poster Presentation).

Interuniversity Stem Cell Meeting, 20th April 2015, Leuven, Belgium. Infection of stem cell-derived hepatocytes with the hepatitis E virus. (Poster presentation).

Hemibio, Annual Consortium Meeting 3, 14-15 January 2015, Chur, Switzerland. Optimization of PSC derived hepatocyte differentiation and epigenetic profile of stem cell-derived hepatocytes. (Oral presentation).

1st Meeting of the Belgian Society for Stem Cell Research (BeSSCR), 12 September 2014, Gent, Belgium. Stem cell-derived hepatocytes as a novel *in vitro* model to study hepatotropic viruses. (Poster presentation).

49th Annual meeting of the European association for the study of the liver (EASL), 9-13 April 2014, London, United Kingdom. Stem cell-derived hepatocytes as a novel *in vitro* model to study hepatotropic viruses. (Poster presentation).

SEURAT-1 Annual Meeting, 5-6 Febraury 2014, Barcelona, Spain. Infection of stem cell-derived hepatocytes with hepatotropic viruses. (Poster presentation).

Hemibio, Annual Consortium Meeting 2, 23-24 January 2014, Liver progeny from pluripotent stem cells. (Oral presentation).